REAGENTS AND METHODS FOR DETECTING SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application Nos. 60/464,115, filed April 21, 2003; 60/464,345, filed April 22, 2003; 60/464,643, filed April 23, 2003; 60/464,965, filed April 24, 2003; and 60/496,016, filed August 19, 2003, the disclosures of which are each incorporated by reference in their entirety for all purposes.

FIELD OF THE INVENTION

[0002] The present invention relates to the fields of molecular biology and nucleic acid chemistry. The invention provides methods and reagents for detecting the severe acute respiratory syndrome coronavirus (SARS CoV) and accordingly, also relates to the fields of medical diagnostics and prognostics.

BACKGROUND OF THE INVENTION

[0003] Severe acute respiratory syndrome (SARS) was first identified in late November 2002 in Guangdong Province, China. In the ensuing months, major outbreaks were reported in other parts of China, Vietnam, Canada, Singapore, Taiwan, and elsewhere in the world. The disease is unusual in its high level of infectivity, as demonstrated among the health care workers and family members that have been in close contact with infected individuals. In addition, it has also been reported that infected patients do not respond to empirical antimicrobial treatment for acute community-acquired typical or atypical pneumonia (Peiris et al. (2003) "Coronavirus as a possible cause of severe acute respiratory syndrome," Lancet 361:1319–1325, which is incorporated by reference).

[0004] The cause of SARS has been identified as a novel coronavirus (CoV) (Drosten et al. (2003) "Identification of a novel coronavirus in patients with severe acute respiratory syndrome," N. Engl. J. Med. 348:1967–1976, which is incorporated by reference), because clinical specimens from patients infected with SARS revealed the presence of crownshaped CoV particles. This new CoV has thus been referred to as

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SARS CoV. The full-length genome sequence of the SARS CoV has been reported from different isolates, and the genome organization of SARS CoV was found to be similar to that of other CoVs (Marra et al. (2003) "The genome sequence of the SARS-associated coronavirus," Science 300:1399–1404 and Ruan et al. (2003) "Comparative full-length genome sequence analysis of 14 SARS coronavirus isolates and common mutations associated with putative origins of infection," Lancet 361:1779–1785, which are both incorporated by reference).

[0005] CoVs are a family of positive-strand RNA-enveloped viruses called *Coronaviridae*, which are now categorized under the newly established order *Nidovirales*. This order comprises the families *Coronaviridae* and *Arteriviridae*. The name *Nidovirales* comes from the Latin word nidus, for nest, referring to the 3'-coterminal "nested" set of subgenomic mRNAs produced during viral infection (Cavanagh (2003) "*Nidovirales*: a new order comprising *Coronaviridae* and *Arteriviridae*," Arch. Virol. 14:629–633, which is incorporated by reference). The SARS CoV genome is very large, 29.7 kb (Marra et al. (2003), *supra*, and Ruan et al. (2003), *supra*, which are both incorporated by reference), and encodes 23 putative proteins. Major structural proteins include nucleocapsid, spike, membrane, and small envelope. Nonstructural proteins include the papainlike proteinase, 3C-like proteinase, RNA-dependent RNA polymerase (RdRp), helicase, and many other proteins involved with viral replication and transcription (Cavanagh (2003), *supra*, and Ng et al. (2002) "Membrane association and dimerization of a cysteine-rich, 16-kilodalton polypeptide released from the C-terminal region of the coronavirus infectious bronchitis virus 1a polyprotein," J. Virol. 76:6257–6267, which are both incorporated by reference). In other CoVs, many of the nonstructural proteins are only slightly conserved in the viral sequence, the exception being RdRp, which is highly conserved in many CoVs.

[0006] The use of oligonucleotide sequences as primers and/or probes for the recognition of infectious agents is one alternative to problematic immunological identification assays and other pre-existing methodologies. For example, nucleic acid probes complementary to targeted nucleic acid sequences have been used in hybridization procedures, such as Southern blots and dot blots, to detect the target nucleic acid sequence. Many of these hybridization procedures have depended on the

cultivation and/or enrichment of the organism and, thus, are generally unsuitable for rapid diagnosis. The advent of techniques for the rapid amplification of specific nucleic acid sequences, such as the polymerase chain reaction among many others, has provided a mechanism to use primer and probe nucleic acids directly on clinical specimens, thereby eliminating enrichment and *in vitro* culturing of the pathogen prior to performing the hybridization assay. Thus, amplification-based hybridization assays can provide simple and rapid diagnostic techniques for the detection of pathogens, such as the SARS CoV in clinical samples.

SUMMARY OF THE INVENTION

[0007] The present invention provides methods and reagents for the rapid, reliable, and highly sensitive detection of the severe acute respiratory syndrome coronavirus without substantial detection of, or cross-reactivity with, other species in the coronavirus genus or species from other genera. Further, the methods and reagents of the invention can be utilized to detect the virus independent of the particular SARS CoV viral type. In addition to oligonucleotides, compositions, and reaction mixtures, the invention also relates to kits and systems for detecting these pathogenic agents, and to related computer and computer readable media.

[0008] In one aspect, the invention provides an oligonucleotide consisting of a nucleic acid with a sequence selected from the group consisting of: SEQ ID NOS: 1-12 and 15-24 or complements thereof. In another aspect, the invention provides an oligonucleotide comprising a nucleic acid with a sequence selected from the group consisting of: SEQ ID NOS: 1-12 and 15-24 and complements thereof, which oligonucleotide has 100 or fewer nucleotides. In still another aspect, the invention provides an oligonucleotide that includes a nucleic acid having at least 90% sequence identity (e.g., at least 95%, etc.) to one of SEQ ID NOS: 1-12 and 15-24 or a complement thereof, which oligonucleotide has 100 or fewer nucleotides. Typically, these oligonucleotides are primer nucleic acids, probe nucleic acids, or the like in these embodiments. In certain embodiments, the oligonucleotides described herein have sequences between about 12 and about 50 nucleotides in length. For example, these oligonucleotides have 40 or fewer nucleotides (e.g., 35 or fewer nucleotides, 30 or fewer nucleotides, etc.) in some embodiments. To further illustrate, the

oligonucleotides comprise at least one modified nucleotide in some embodiments. In certain embodiments, for example, at least one nucleotide of an oligonucleotide described herein is modified to alter nucleic acid hybridization stability relative to unmodified nucleotides. In some embodiments, the oligonucleotides described herein comprise at least one label and/or at least one quencher moiety. In certain embodiments, the oligonucleotides include at least one conservatively modified variation.

[0009] In another aspect, the invention relates to a method of detecting a severe acute respiratory syndrome coronavirus in a sample. The method includes (a) contacting nucleic acids from the sample with at least one primer nucleic acid comprising at least one nucleic acid selected from the group consisting of: SEQ ID NOS: 1-12 and 15-24 and complements thereof in at least one nucleic acid amplification reaction (e.g., a nested polymerase chain reaction or the like). In addition, the method also includes (b) detecting the nucleic acids and/or one or more amplicons thereof from the nucleic acid amplification reaction during or after (a), thereby detecting the severe acute respiratory syndrome coronavirus in the sample. The sample is typically derived from a mammalian subject, such as a human subject. In certain embodiments, at least one of the amplicons is about 440 nucleotides in length. In some embodiments, at least one round of the nucleic acid amplification reaction is performed using primer nucleic acids comprising sequences selected from SEQ ID NOS: 11 or 22 and SEQ ID NOS: 12 or 20. In certain embodiments, at least one round of the nucleic acid amplification reaction is performed using primer nucleic acids comprising sequences selected from SEQ ID NOS: 15 or 18 and SEQ ID NOS: 16 or 19. Optionally, at least one of the primer nucleic acids comprises a modified primer nucleic acid and/or comprises at least one label. In some embodiments, for example, the modified primer nucleic acid comprises a nucleic acid amplification specificity altering modification and/or a restriction site linker modification. In certain embodiments, (b) comprises monitoring binding between the amplicons and at least one oligonucleotide having a sequence selected from the group consisting of: SEQ ID NOS: 1-12 and 15-24, a substantially identical variant thereof wherein the variant has at least 90% sequence identity to one of SEQ ID NOS: 1-12 and 15-24, and complements of SEQ ID NOS: 1-12 and 15-24 and the variant. Typically, the oligonucleotide

comprises at least one label and/or at least one quencher moiety. In certain embodiments, for example, the oligonucleotide comprises a 5'-nuclease probe having a sequence selected from SEQ ID NO: 27 or SEQ ID NO: 28. In these embodiments, (b) optionally comprises detecting a detectable signal produced by the label, or amplifying a detectable signal produced by the label to produce an amplified signal and detecting the amplified signal.

[0010] In still another aspect, the invention provides a method of determining a presence of a severe acute respiratory syndrome coronavirus in a sample, which method comprises (a) contacting nucleic acids and/or amplicons thereof from the sample with one or more oligonucleotides that comprise at least one nucleic acid with a sequence selected from the group consisting of: SEQ ID NOS: 1-12 and 15-24, a substantially identical variant thereof wherein the variant has at least 90% sequence identity to one of SEQ ID NOS: 1-12 and 15-24, and complements of SEQ ID NOS: 1-12 and 15-24 and the variant. The method also includes (b) monitoring (e.g., at a single time point, at multiple discrete time points, continuously over a selected time period, etc.) binding between the nucleic acids and/or amplicons thereof, and the oligonucleotides; in which detectable binding between the nucleic acids and/or amplicons thereof, and the oligonucleotides, determines the presence of the severe acute respiratory syndrome coronavirus in the sample. The presence of the severe acute respiratory syndrome coronavirus in the sample is generally unknown or unsubstantiated before (a). In certain embodiments, (a) comprises contacting the nucleic acids and/or amplicons thereof with the oligonucleotides in solution at a temperature of at least 42°C for at least 15 minutes in which a total weight of the solution comprises about 50% formalin and comprises heparin at a concentration of about 1 mg/ml. Moreover, the method typically comprises a reaction other than a sequencing reaction. The sample is generally derived from a mammalian subject, such as a human subject. In certain embodiments, the nucleic acids and/or amplicons thereof and the oligonucleotides are contacted in solution. Optionally, a solid support comprises the nucleic acids and/or amplicons (e.g., arrayed on the solid support). As an additional option, a solid support comprises the oligonucleotides.

[0011] In some embodiments, at least one segment of the nucleic acids is amplified prior to or during (a) using at least one nucleic acid amplification technique to produce the amplicons and (b) comprises monitoring the binding between the nucleic acids and/or amplicons thereof, and the oligonucleotides, during or after amplification. For example, the nucleic acid amplification technique typically comprises a polymerase chain reaction (e.g., a nest polymerase chain reaction, etc.), a ligase chain reaction, and/or the like. In these embodiments, the segment is optionally amplified using at least one primer nucleic acid comprising a sequence selected from the group consisting of: SEQ ID NOS: 1-12 and 15-24, a substantially identical variant thereof in which the variant has at least 90% sequence identity to one of SEQ ID NOS: 1-12 and 15-24, and complements of SEQ ID NOS: 1-12 and 15-24 and the variant. In some of these embodiments, the primer nucleic acid comprises at least one label, as described herein or otherwise known in the art. Optionally, the primer nucleic acid comprises a modified primer nucleic acid (e.g., a nucleic acid amplification specificity altering modification, a restriction site linker, and/or the like).

[0012] In another aspect, the invention relates to a composition comprising a sample derived from a subject and at least one oligonucleotide that comprises a nucleic acid with a sequence selected from the group consisting of: SEQ ID NOS: 1-12 and 15-24, a substantially identical variant thereof wherein the variant has at least 90% sequence identity to one of SEQ ID NOS: 1-12 and 15-24, and complements of SEQ ID NOS: 1-12 and 15-24 and the variant, which oligonucleotide consists of 100 or fewer nucleotides. A presence of a severe acute respiratory syndrome coronavirus in the sample is generally unknown or unsubstantiated. Typically, the oligonucleotides comprise at least one chemically synthesized nucleic acid. In certain embodiments, at least one nucleotide of a given oligonucleotide is modified (e.g., to alter nucleic acid hybridization stability relative to unmodified nucleotides).

[0013] Typically, at least one of the oligonucleotides comprises at least one label and/or at least one quencher moiety. To illustrate, the label optionally comprises a fluorescent dye, a weakly fluorescent label, a non-fluorescent label, a colorimetric label, a chemiluminescent label, a bioluminescent label, an antibody, an antigen, biotin, a hapten, a mass-modifying group, a radioisotope, an enzyme, or the like.

[0014] The oligonucleotides of the compositions of the invention are provided in various formats. In some embodiments, for example, at least one of the oligonucleotides is in solution. In other embodiments, a solid support comprises at least one of the oligonucleotides. In these embodiments, the oligonucleotides are non-covalently or covalently attached to the solid support. Exemplary solid supports utilized in these embodiments are optionally selected from, e.g., a plate, a microwell plate, a bead, a microbead (e.g., a magnetic microbead, etc), a tube (e.g., a microtube, etc.), a fiber, a whisker, a comb, a hybridization chip, a membrane, a single crystal, a ceramic layer, a self-assembling monolayer, and the like.

[0015] To further illustrate, the oligonucleotides are optionally conjugated with biotin or a biotin derivative and the solid support is optionally conjugated with avidin or an avidin derivative, or streptavidin or a streptavidin derivative. In some embodiments, a linker attaches the oligonucleotides to the solid support. The linker is typically selected from, e.g., an oligopeptide, an oligonucleotide, an oligopolyamide, an oligoethyleneglycerol, an oligoacrylamide, an alkyl chain, and the like. Optionally, a cleavable attachment attaches the oligonucleotides to the solid support. The cleavable attachment is generally cleavable by, e.g., heat, an enzyme, a chemical agent, electromagnetic radiation, etc.

[0016] In another aspect, the invention provides a kit that includes (a) at least one oligonucleotide that comprises a nucleic acid with a sequence selected from the group consisting of: SEQ ID NOS: 1-12 and 15-24, a substantially identical variant thereof wherein the variant has at least 90% sequence identity to one of SEQ ID NOS: 1-12 and 15-24, and complements of SEQ ID NOS: 1-12 and 15-24 and the variant, which oligonucleotide consists of 100 or fewer nucleotides; and one or more of: (b) instructions for determining a presence of a severe acute respiratory syndrome coronavirus in a sample by monitoring binding between nucleic acids and/or amplicons thereof from the sample and the oligonucleotide in which the presence of a severe acute respiratory syndrome coronavirus in the sample is unknown or unsubstantiated, or (c) at least one container for packaging at least the oligonucleotide. In some embodiments, the kit further includes at least one enzyme (e.g., a polymerase, etc.) and/or one or more nucleotides (e.g., deoxyribonucleotides, etc.).

[0017] In some embodiments, the oligonucleotide is in solution, whereas in others, a solid support comprises the oligonucleotide. The solid support is optionally selected from, e.g., a plate, a microwell plate, a bead, a microbead, a tube, a fiber, a whisker, a comb, a hybridization chip, a membrane, a single crystal, a ceramic layer, a self-assembling monolayer, or the like.

[0018] Typically, the oligonucleotide comprises at least one label and/or at least one quencher moiety. Exemplary labels include, e.g., a fluorescent dye, a weakly fluorescent label, a non-fluorescent label, a colorimetric label, a chemiluminescent label, a bioluminescent label, an antibody, an antigen, biotin, a hapten, a mass-modifying group, a radioisotope, an enzyme, or the like.

[0019] In still other aspects, the invention provides a system (e.g., an automated system) for detecting a severe acute respiratory syndrome coronavirus in a sample. The system includes (a) at least one oligonucleotide that comprises a nucleic acid with a sequence selected from the group consisting of: SEQ ID NOS: 1-12 and 15-24, a substantially identical variant thereof wherein the variant has at least 90% sequence identity to one of SEQ ID NOS: 1-12 and 15-24, and complements of SEQ ID NOS: 1-12 and 15-24 and the variant, which oligonucleotide consists of 100 or fewer nucleotides. The system also includes (b) at least one detector that detects binding between nucleic acids and/or amplicons thereof from the sample and the oligonucleotide, and (c) at least one controller operably connected to the detector, which controller comprises one or more instructions sets that correlate the binding detected by the detector with a presence of the severe acute respiratory syndrome coronavirus in the sample. In addition, the oligonucleotide typically comprises at least one label and/or at least one quencher moiety. In some embodiments, at least one container or solid support comprises the oligonucleotide. In these embodiments, the system optionally further includes (d) at least one thermal modulator operably connected to the container or solid support to modulate temperature in the container or on the solid support, and/or (e) at least one fluid transfer component that transfers fluid to and/or from the container or solid support, e.g., for performing one or more nucleic acid amplification techniques in the container or on the solid support, etc.

[0020] In yet another aspect, the invention provides a system that includes (a) computer or computer readable medium comprising a data set that comprises a plurality of character strings that correspond to a plurality of sequences that correspond to one or more of: SEQ ID NOS: 1-12 and 15-24, a substantially identical variant thereof wherein the variant has at least 90% sequence identity to one of SEQ ID NOS: 1-12 and 15-24, and complements of SEQ ID NOS: 1-12 and 15-24 and the variant. The system also includes (b) an automatic synthesizer coupled to an output of the computer or computer readable medium, which automatic synthesizer accepts instructions from the computer or computer readable medium, which instructions direct synthesis of one or more nucleic acids that correspond to one or more character strings in the data set.

APPENDIX

[0021] This application is being filed with a paper appendix totaling 22 pages. This appendix provides additional description of certain aspects of the invention and is incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] Figure 1 shows a nucleotide sequence of the SARS-CoV genome as determined by the Centers for Disease Control (Atlanta, GA, USA).

[0023] Figure 2 shows alignments of nucleotide sequences from various SARS-CoV isolates and from certain non-target organisms relative to one another.

[0024] Figure 3 schematically depicts a nested PCR procedure for detecting the SARS-CoV in a sample.

[0025] Figure 4 is a block diagram showing a representative example system for detecting the SARS-CoV in a sample.

[0026] Figure 5 is a block diagram showing a representative example system including a computer and a computer readable medium in which various aspects of the present invention may be embodied.

[0027] Figure 6 is a photograph of an agarose gel that shows the results of a nested polymerase chain reaction in a positive clinical sample according to the procedure of Example 2 using primers of SEQ ID NO: 11 and SEQ ID NO: 12 as

reagents 6 and 7 and using primers of SEQ ID NO: 13 and SEQ ID NO: 14 as reagents 8 and 9, respectively.

DETAILED DESCRIPTION

I. DEFINITIONS

[0028] Before describing the present invention in detail, it is to be understood that this invention is not limited to particular oligonucleotides (e.g., primer nucleic acids, probe nucleic acids, etc.), methods, compositions, reaction mixtures, kits, systems, computers, or computer readable media, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. Further, unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In describing and/or claiming the present invention, the following terminology and grammatical variants will be used in accordance with the definitions set forth below.

[0029] A "**5'-nuclease probe**" refers to an oligonucleotide that comprises at least two labels and emits radiation of increased intensity after one of the two labels is cleaved or otherwise separated from the probe. In certain embodiments, for example, a 5'-nuclease probe is labeled with two different fluorescent dyes, e.g., a 5' terminus reporter dye and the 3' terminus quenching dye or moiety. When the probe is intact, energy transfer typically occurs between the two fluorophores such that fluorescent emission from the reporter dye is quenched. During an extension step of a polymerase chain reaction, for example, a 5'-nuclease probe bound to a template nucleic acid is cleaved by the 5' nuclease activity of, e.g., a Taq polymerase such that the fluorescent emission of the reporter dye is no longer quenched. Exemplary 5'-nuclease probes are described in, e.g., U.S. Pat. No. 5,210,015, entitled "HOMOGENEOUS ASSAY SYSTEM USING THE NUCLEASE ACTIVITY OF A NUCLEIC ACID POLYMERASE," issued May 11, 1993 to Gelfand et al., U.S. Pat. No. 5,994,056, entitled "HOMOGENEOUS METHODS FOR NUCLEIC ACID AMPLIFICATION AND DETECTION," issued November 30, 1999 to Higuchi, and U.S. Pat. No. 6,171,785, entitled "METHODS AND DEVICES FOR HEMOGENEOUS NUCLEIC

ACID AMPLIFICATION AND DETECTOR," issued January 9, 2001 to Higuchi, which are each incorporated by reference.

[0030] The term "**alteration**" refers to a change in a nucleic acid sequence, including, but not limited to, a substitution, an insertion, and/or a deletion.

[0031] An "**amplification reaction**" refers to a primer initiated replication of one or more target nucleic acid sequences or complements thereto.

[0032] An "**amplicon**" refers to a molecule made by copying or transcribing another molecule, e.g., as occurs in transcription, cloning, and/or in a polymerase chain reaction ("PCR") (e.g., nested PCR, strand displacement PCR amplification (SDA), duplex PCR amplification, etc.) or another nucleic acid amplification technique. Typically, an amplicon is a copy of a selected nucleic acid (e.g., a template or target nucleic acid) or is complementary thereto.

[0033] An "**amplified signal**" refers to increased detectable signal that can be produced in the absence of, or in conjunction with, an amplification reaction. Exemplary signal amplification techniques are described in, e.g., Cao et al. (1995) "Clinical evaluation of branched DNA signal amplification for quantifying HIV type 1 in human plasma," AIDS Res Hum Retroviruses 11(3):353-361, and in U.S Pat. No. 5,437,977 to Segev, U.S. Pat. No. 6,033,853 to Delair et al., and U.S. Pat. No. 6,180,777 to Horn, which are each incorporated by reference.

[0034] "**Antibody**" refers to a polypeptide substantially encoded by at least one immunoglobulin gene or fragments of at least one immunoglobulin gene, that can participate in detectable binding with a ligand. The term includes naturally-occurring forms, as well as fragments and derivatives. Fragments within the scope of the term as used herein include those produced by digestion with various peptidases, such as Fab, Fab' and F(ab)'2 fragments, those produced by chemical dissociation, by chemical cleavage, so long as the fragment remains capable of detectable binding to a target molecule, such as an antigen indicative of a disease.

[0035] An "**array**" refers to an assemblage of elements. The assemblage can be spatially ordered (a "patterned array") or disordered (a "randomly patterned" array). The array can form or comprise one or more functional elements (e.g., a probe region on a microarray) or it can be non-functional.

[0036] The term "**attached**" or "**conjugated**" refers to interactions and/or states in which material or compounds are connected or otherwise joined with one another. These interactions and/or states are typically produced by, e.g., covalent bonding, ionic bonding, chemisorption, physisorption, and combinations thereof. In certain embodiments, for example, oligonucleotides are attached to solid supports. In some of these embodiments, an oligonucleotide is conjugated with biotin (i.e., is biotinylated) and a solid support is conjugated with avidin such that the oligonucleotide attaches to the solid support via the binding interaction of, e.g., biotin and avidin.

[0037] Molecular species "**bind**" when they associate with one another via covalent and/or non-covalent interactions. For example, two complementary single-stranded nucleic acids can hybridize with one another to form a nucleic acid with at least one double-stranded region. To further illustrate, antibodies and corresponding antigens can also non-covalently associate with one another.

[0038] The term "**cleavage**" refers to a process of releasing a material or compound from attachment to another material or compound. In certain embodiments, for example, oligonucleotides are cleaved from, e.g., a solid support to permit analysis of the oligonucleotides by solution-phase methods. *See, e.g.,* Wells et al. (1998) "Cleavage and Analysis of Material from Single Resin Beads," J. Org. Chem. 63:6430, which is incorporated by reference.

[0039] A "**character**" when used in reference to a character of a character string refers to a subunit of the string. In one embodiment, the character of a character string encodes one subunit of an encoded biological molecule. Thus, for example, where the encoded biological molecule is a polynucleotide or oligonucleotide, a character of the string encodes a single nucleotide.

[0040] A "**character string**" is any entity capable of storing sequence information (e.g., the subunit structure of a biological molecule such as the nucleotide sequence of a nucleic acid, etc.). In one embodiment, the character string can be a simple sequence of characters (letters, numbers, or other symbols) or it can be a numeric or coded representation of such information in tangible or intangible (e.g., electronic, magnetic, etc.) form. The character string need not be "linear," but can also exist in a number of other forms, e.g., a linked list or other non-linear array (e.g., used as a code to generate a linear array of characters), or the like. Character strings are

typically those which encode oligonucleotide or polynucleotide strings, directly or indirectly, including any encrypted strings, or images, or arrangements of objects which can be transformed unambiguously to character strings representing sequences of monomers or multimers in polynucleotides, or the like (whether made of natural or artificial monomers).

[0041] The term "**complement thereof**" refers to nucleic acid that is both the same length as, and exactly complementary to, a given nucleic acid.

[0042] A "**composition**" refers to a combination of two or more different components. In certain embodiments, for example, a composition includes a solid support that comprises one or more oligonucleotides, e.g., covalently or non-covalently attached to a surface of the support. In other embodiments, a composition includes one or more oligonucleotides in solution.

[0043] The term "**deletion**" in the context of a nucleic acid sequence refers to an alteration in which at least one nucleotide is removed from the nucleic acid sequence, e.g., from a 5'-terminus, from a 3'-terminus, and/or from an internal position of the nucleic acid sequence.

[0044] The term "**derivative**" refers to a chemical substance related structurally to another substance, or a chemical substance that can be made from another substance (i.e., the substance it is derived from), e.g., through chemical or enzymatic modification. To illustrate, oligonucleotides are optionally conjugated with biotin or a biotin derivative. To further illustrate, one nucleic acid can be "derived" from another through processes, such as chemical synthesis based on knowledge of the sequence of the other nucleic acid, amplification of the other nucleic acid, or the like.

[0045] The term "**detectably bind**" refers to binding between at least two molecular species (e.g., a probe nucleic acid and a target nucleic acid, a sequence specific antibody and a target nucleic acid, etc.) that is detectable above a background signal (e.g., noise) using one or more methods of detection.

[0046] Nucleic acids are "**extended**" or "**elongated**" when additional nucleotides (or other analogous molecules) are incorporated into the nucleic acids. For example, a nucleic acid is optionally extended by a nucleotide incorporating

biocatalyst, such as a polymerase that typically adds nucleotides at the 3' terminal end of a nucleic acid.

[0047] An "**extended primer nucleic acid**" refers to a primer nucleic acid to which one or more additional nucleotides have been added or otherwise incorporated (e.g., covalently bonded thereto).

[0048] Nucleic acids "**hybridize**" or "**bind**" when they associate with one another, typically in solution. Nucleic acids hybridize due to a variety of well characterized physico-chemical forces, such as hydrogen bonding, solvent exclusion, base stacking and the like. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes part I chapter 2, "Overview of principles of hybridization and the strategy of nucleic acid probe assays," (Elsevier, New York), as well as in Ausubel (Ed.) Current Protocols in Molecular Biology, Volumes I, II, and III, 1997, which is incorporated by reference. Hames and Higgins (1995) Gene Probes 1 IRL Press at Oxford University Press, Oxford, England, (Hames and Higgins 1) and Hames and Higgins (1995) Gene Probes 2 IRL Press at Oxford University Press, Oxford, England (Hames and Higgins 2) provide details on the synthesis, labeling, detection and quantification of DNA and RNA, including oligonucleotides. Both Hames and Higgins 1 and 2 are incorporated by reference.

[0049] "**Stringent hybridization wash conditions**" in the context of nucleic acid hybridization assays or experiments, such as nucleic acid amplification reactions, Southern and northern hybridizations, or the like, are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993), *supra*. and in Hames and Higgins, 1 and 2.

[0050] For purposes of the present invention "**highly stringent**" hybridization and wash conditions are selected to be at least about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the test sequence hybridizes to a perfectly matched oligonucleotide. Very stringent conditions are selected to be equal to the T_m for a particular oligonucleotide.

[0051] An example of stringent hybridization conditions for hybridization of complementary nucleic acids on a filter in a Southern or northern blot is 50% formalin with 1 mg of heparin at 42°C, with the hybridization being carried out overnight. An example of stringent wash conditions is a 0.2x SSC wash at 65°C for 15 minutes (*see,* Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2001), which is incorporated by reference, for a description of SSC buffer). Often the high stringency wash is preceded by a low stringency wash to remove background probe signal. An example low stringency wash is 2x SSC at 40°C for 15 minutes. In general, a signal to noise ratio of 5x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization.

[0052] Comparative hybridization can be used to identify nucleic acids of the invention.

[0053] In particular, detection of stringent hybridization in the context of the present invention indicates strong structural similarity to, e.g., the nucleic acids provided in the sequence listing herein. For example, it is desirable to identify test nucleic acids that hybridize to the exemplar nucleic acids herein under stringent conditions. One measure of stringent hybridization is the ability to detectably hybridize to one of the listed nucleic acids (e.g., nucleic acids with sequences selected from SEQ ID NOS: 1-12 and 15-24 and complements thereof) under stringent conditions. Stringent hybridization and wash conditions can easily be determined empirically for any test nucleic acid.

[0054] For example, in determining highly stringent hybridization and wash conditions, the stringency of the hybridization and wash conditions are gradually increased (e.g., by increasing temperature, decreasing salt concentration, increasing detergent concentration and/or increasing the concentration of organic solvents such as formalin in the hybridization or wash), until a selected set of criteria is met. For example, the stringency of the hybridization and wash conditions are gradually increased until a oligonucleotide consisting of or comprising one or more nucleic acid sequences selected from SEQ ID NOS: 1-12 and 15-24 and complementary polynucleotide sequences thereof, binds to a perfectly matched complementary target (again, a nucleic acid comprising one or more nucleic acid sequences selected from

SEQ ID NOS: 1-12 and 15-24 and complementary polynucleotide sequences thereof), with a signal to noise ratio that is at least 5x as high as that observed for hybridization of the oligonucleotide to non-target nucleic acids present in the sample (e.g., nucleic acids from organisms other than SARS-CoV).

[0055] The detection of target nucleic acids which hybridize to the nucleic acids represented by SEQ ID NOS: 1-12 and 15-24 under high stringency conditions are a feature of the invention. Examples of such nucleic acids include those with one or a few silent or conservative nucleic acid substitutions as compared to a given nucleic acid sequence.

[0056] The terms "**identical**" or percent "**identity**" in the context of two or more nucleic acid or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, e.g., as measured using one of the sequence comparison algorithms available to persons of skill or by visual inspection. Exemplary algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST programs, which are described in, e.g., Altschul et al. (1990) "Basic local alignment search tool" J. Mol. Biol. 215:403-410, Gish et al. (1993) "Identification of protein coding regions by database similarity search" Nature Genet. 3:266-272, Madden et al. (1996) "Applications of network BLAST server" Meth. Enzymol. 266:131-141, Altschul et al. (1997) "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs" Nucleic Acids Res. 25:3389-3402, and Zhang et al. (1997) "PowerBLAST: A new network BLAST application for interactive or automated sequence analysis and annotation" Genome Res. 7:649-656, which are each incorporated by reference. Many other optimal alignment algorithms are also known in the art and are optionally utilized to determine percent sequence identity.

[0057] The phrase "**in solution**" refers to an assay or reaction condition in which the components of the assay or reaction are not attached to a solid support and are present in a liquid medium. Exemplary liquid mediums include aqueous and organic fluids. For example, certain assays of the invention include incubating oligonucleotides together with SARS-CoV nucleic acids and SARS-CoV nucleic acid amplicons in solution to allow hybridization to occur.

[0058] The term "**insertion**" in the context of a nucleic acid sequence refers to an alteration in which at least one nucleotide is added to the nucleic acid sequence, e.g., at a 5'-terminus, at a 3'-terminus, and/or at an internal position of the nucleic acid sequence.

[0059] A "**label**" refers to a moiety attached (covalently or non-covalently), or capable of being attached, to a molecule, which moiety provides or is capable of providing information about the molecule (e.g., descriptive, identifying, etc. information about the molecule) or another molecule with which the labeled molecule interacts (e.g., hybridizes, etc.). Exemplary labels include fluorescent labels (including, e.g., quenchers or absorbers), weakly fluorescent labels, non-fluorescent labels, colorimetric labels, chemiluminescent labels, bioluminescent labels, radioactive labels, mass-modifying groups, antibodies, antigens, biotin, haptens, enzymes (including, e.g., peroxidase, phosphatase, etc.), and the like.

[0060] A "**linker**" refers to a chemical moiety that covalently or non-covalently attaches a compound or substituent group to another moiety, e.g., a probe nucleic acid, a primer nucleic acid, an amplicon, a solid support, or the like. For example, linkers are optionally used to attach oligonucleotides to a solid support (e.g., in a linear or other logic probe array). To further illustrate, a linker optionally attaches a label (e.g., a fluorescent dye, a radioisotope, etc.) to a probe nucleic acid, a primer nucleic acid, or the like. Linkers are typically at least bifunctional chemical moieties and in certain embodiments, they comprise cleavable attachments, which can be cleaved by, e.g., heat, an enzyme, a chemical agent, electromagnetic radiation, etc. to release materials or compounds from, e.g., a solid support. A careful choice of linker allows cleavage to be performed under appropriate conditions compatible with the stability of the compound and assay method. Generally a linker has no specific biological activity other than to, e.g., join chemical species together or to preserve some minimum distance or other spatial relationship between such species. However, the constituents of a linker may be selected to influence some property of the linked chemical species such as three-dimensional conformation, net charge, hydrophobicity, etc. Exemplary linkers include, e.g., oligopeptides, oligonucleotides, oligopolyamides, oligoethyleneglycerols, oligoacrylamides, alkyl chains, or the like. Additional description of linker molecules is provided in, e.g., Hermanson, Bioconjugate

Techniques, Elsevier Science (1996), Lyttle et al. (1996) Nucleic Acids Res. 24(14):2793, Shchepino et al. (2001) Nucleosides, Nucleotides, & Nucleic Acids 20:369, Doronina et al (2001) Nucleosides, Nucleotides, & Nucleic Acids 20:1007, Trawick et al. (2001) Bioconjugate Chem. 12:900, Olejnik et al. (1998) Methods in Enzymology 291:135, and Pljevaljcic et al. (2003) J. Am. Chem. Soc. 125(12):3486, all of which are incorporated by reference.

[0061] A "**mass modifying**" group modifies the mass, typically measured in terms of molecular weight as daltons, of a molecule that comprises the group. For example, mass modifying groups that increase the discrimination between at least two nucleic acids with single base differences in size or sequence can be used to facilitate sequencing using, e.g., molecular weight determinations.

[0062] A "**mixture**" refers to a combination of two or more different components. A "**reaction mixture**" refers a mixture that comprises molecules that can participate in and/or facilitate a given reaction. An "**amplification reaction mixture**" refers to a solution containing reagents necessary to carry out an amplification reaction, and typically contains primers, a thermostable DNA polymerase, dNTP's, and a divalent metal cation in a suitable buffer. A reaction mixture is referred to as complete if it contains all reagents necessary to carry out the reaction, and incomplete if it contains only a subset of the necessary reagents. It will be understood by one of skill in the art that reaction components are routinely stored as separate solutions, each containing a subset of the total components, for reasons of convenience, storage stability, or to allow for application-dependent adjustment of the component concentrations, and, that reaction components are combined prior to the reaction to create a complete reaction mixture. Furthermore, it will be understood by one of skill in the art that reaction components are packaged separately for commercialization and that useful commercial kits may contain any subset of the reaction components, which includes the modified primers of the invention.

[0063] A "**modified primer nucleic acid**" refers to a primer nucleic acid that comprises a moiety or sequence of nucleotides that provides a desired property to the primer nucleic acid. In certain embodiments, for example, modified primer nucleic acids comprise "**nucleic acid amplification specificity altering modifications**" that, e.g., reduce non-specific nucleic acid amplification (e.g., minimize primer dimer

formation or the like), increase the yield of an intended target amplicon, and/or the like. Examples of nucleic acid amplification specificity altering modifications are described in, e.g., U.S. Pat. No. 6,001,611, entitled "MODIFIED NUCLEIC ACID AMPLIFICATION PRIMERS," issued December 14, 1999 to Will, which is incorporated by reference. Other exemplary primer nucleic acid modifications include a "**restriction site linker modification**" in which a nucleotide sequence comprising a selected restriction site is attached, e.g., at 5'-terminus of a primer nucleic acid. Restriction site linkers are typically attached to primer nucleic acids to facilitate subsequent amplicon cloning or the like.

[0064] A "**moiety**" or "**group**" refers to one of the portions into which something, such as a molecule, is divided (e.g., a functional group, substituent group, or the like). For example, an oligonucleotide optionally comprises a quencher moiety, a labeling moiety, or the like.

[0065] The term "**nucleic acid**" refers to nucleotides (e.g., ribonucleotides, deoxyribonucleotides, dideoxynucleotides, etc.) and polymers that comprise such nucleotides covalently linked together, either in a linear or branched fashion. Exemplary nucleic acids include deoxyribonucleoic acids (DNAs), ribonucleic acids (RNAs), DNA-RNA hybrids, oligonucleotides, polynucleotides, genes, cDNAs, aptamers, antisense nucleic acids, interfering RNAs (RNAis), molecular beacons, nucleic acid probes, peptide nucleic acids (PNAs), locked nucleic acids (LNA™s), PNA-DNA conjugates, PNA-RNA conjugates, LNA™-DNA conjugates, LNA™-RNA conjugates, etc.

[0066] A nucleic acid is typically single-stranded or double-stranded and will generally contain phosphodiester bonds, although in some cases, as outlined herein, nucleic acid analogs are included that may have alternate backbones, including, for example and without limitation, phosphoramide (Beaucage et al. (1993) Tetrahedron 49(10):1925 and references therein; Letsinger (1970) J. Org. Chem. 35:3800; Sprinzl et al. (1977) Eur. J. Biochem. 81:579; Letsinger et al. (1986) Nucl. Acids Res. 14: 3487; Sawai et al. (1984) Chem. Lett. 805; Letsinger et al. (1988) J. Am. Chem. Soc. 110:4470; and Pauwels et al. (1986) Chemica Scripta 26: 1419, which are each incorporated by reference), phosphorothioate (Mag et al. (1991) Nucleic Acids Res. 19:1437; and U.S. Pat. No. 5,644,048, which are both incorporated by reference),

phosphorodithioate (Briu et al. (1989) J. Am. Chem. Soc. 111:2321, which is incorporated by reference), O-methylphophoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press (1992), which is incorporated by reference), and peptide nucleic acid backbones and linkages (see, Egholm (1992) J. Am. Chem. Soc. 114:1895; Meier et al. (1992) Chem. Int. Ed. Engl. 31:1008; Nielsen (1993) Nature 365:566; and Carlsson et al. (1996) Nature 380:207, which are each incorporated by reference). Other analog nucleic acids include those with positively charged backbones (Denpcy et al. (1995) Proc. Natl. Acad. Sci. USA 92:6097, which is incorporated by reference); non-ionic backbones (U.S. Pat. Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Angew (1991) Chem. Intl. Ed. English 30: 423; Letsinger et al. (1988) J. Am. Chem. Soc. 110:4470; Letsinger et al. (1994) Nucleoside & Nucleotide 13:1597; Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y. S. Sanghvi and P. Dan Cook; Mesmaeker et al. (1994) Bioorganic & Medicinal Chem. Lett. 4: 395; Jeffs et al. (1994) J. Biomolecular NMR 34:17; and Tetrahedron Lett. 37:743 (1996), which are each incorporated by reference) and non-ribose backbones, including those described in U.S. Pat. Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, Carbohydrate Modifications in Antisense Research, Ed. Y. S. Sanghvi and P. Dan Cook, which references are each incorporated by reference. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al. (1995) Chem. Soc. Rev. pp169-176, which is incorporated by reference). Several nucleic acid analogs are also described in, e.g., Rawls, C & E News Jun. 2, 1997 page 35, which is incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of additional moieties such as labels, or to alter the stability and half-life of such molecules in physiological environments.

[0067] In addition to these naturally occurring heterocyclic bases that are typically found in nucleic acids (e.g., adenine, guanine, thymine, cytosine, and uracil), nucleic acid analogs also include those having non-naturally occurring heterocyclic or modified bases, many of which are described, or otherwise referred to, herein. In particular, many non-naturally occurring bases are described further in, e.g., Seela et al. (1991) Helv. Chim. Acta 74:1790, Grein et al. (1994) Bioorg. Med. Chem. Lett. 4:971–

976, and Seela et al. (1999) Helv. Chim. Acta 82:1640, which are each incorporated by reference. To further illustrate, certain bases used in nucleotides that act as melting temperature (T_m) modifiers are optionally included. For example, some of these include 7-deazapurines (e.g., 7-deazaguanine, 7-deazaadenine, etc.), pyrazolo[3,4-d]pyrimidines, propynyl-dN (e.g., propynyl-dU, propynyl-dC, etc.), and the like. See, e.g., U.S. Pat. No. 5,990,303, entitled "SYNTHESIS OF 7-DEAZA-2'-DEOXYGUANOSINE NUCLEOTIDES," which issued November 23, 1999 to Seela, which is incorporated by reference. Other representative heterocyclic bases include, e.g., hypoxanthine, inosine, xanthine; 8-aza derivatives of 2-aminopurine, 2,6-diaminopurine, 2-amino-6-chloropurine, hypoxanthine, inosine and xanthine; 7-deaza-8-aza derivatives of adenine, guanine, 2-aminopurine, 2,6-diaminopurine, 2-amino-6-chloropurine, hypoxanthine, inosine and xanthine; 6-azacytosine; 5-fluorocytosine; 5-chlorocytosine; 5-iodocytosine; 5-bromocytosine; 5-methylcytosine; 5-propynylcytosine; 5-bromovinyluracil; 5-fluorouracil; 5-chlorouracil; 5-iodouracil; 5-bromouracil; 5-trifluoromethyluracil; 5-methoxymethyluracil; 5-ethynyluracil; 5-propynyluracil, and the like.

[0068] Examples of modified bases and nucleotides are also described in, e.g., U.S. Pat. No. 5,484,908, entitled "OLIGONUCLEOTIDES CONTAINING 5-PROPYNYL PYRIMIDINES," issued January 16, 1996 to Froehler et al., U.S. Pat. No. 5,645,985, entitled "ENHANCED TRIPLE-HELIX AND DOUBLE-HELIX FORMATION WITH OLIGOMERS CONTAINING MODIFIED PYRIMIDINES," issued July 8, 1997 to Froehler et al., U.S. Pat. No. 5,830,653, entitled "METHODS OF USING OLIGOMERS CONTAINING MODIFIED PYRIMIDINES," issued November 3, 1998 to Froehler et al., U.S. Pat. No. 6,639,059, entitled "SYNTHESIS OF [2.2.1]BICYCLO NUCLEOSIDES," issued October 28, 2003 to Kochkine et al., U.S. Pat. No. 6,303,315, entitled "ONE STEP SAMPLE PREPARATION AND DETECTION OF NUCLEIC ACIDS IN COMPLEX BIOLOGICAL SAMPLES," issued October 16, 2001 to Skouv, and U.S. Pat. Application Pub. No. 2003/0092905, entitled "SYNTHESIS OF [2.2.1]BICYCLO NUCLEOSIDES," by Kochkine et al. that published May 15, 2003, which are each incorporated by reference.

[0069] The term "**nucleic acid detection reagent**" refers to a reagent that detectably binds (e.g., hydrogen bonds in nucleic acid hybridization, in antibody-

antigen recognition, or the like, or other types of binding interactions) to a SARS-CoV nucleic acid. For example, nucleic acids (e.g., probe nucleic acids, primer nucleic acids, etc.) that comprise sequences selected from SEQ ID NOS: 1-12 and 15-24 or complements thereof bind to SARS-CoV nucleic acids. Other exemplary nucleic acid detection reagents include sequence specific antibodies that specifically bind to SARS-CoV nucleic acids.

[0070] A "**nucleotide**" refers to an ester of a nucleoside, e.g., a phosphate ester of a nucleoside. For example, a nucleotide can include 1, 2, 3, or more phosphate groups covalently linked to a 5' position of a sugar moiety of the nucleoside.

[0071] A "**nucleotide incorporating biocatalyst**" refers to a catalyst that catalyzes the incorporation of nucleotides into a nucleic acid. Nucleotide incorporating biocatalysts are typically enzymes. An "**enzyme**" is a protein- and/or nucleic acid-based catalyst that acts to reduce the activation energy of a chemical reaction involving other compounds or "substrates." A "**nucleotide incorporating enzyme**" refers to an enzyme that catalyzes the incorporation of nucleotides into a nucleic acid, e.g., during nucleic acid amplification or the like. Exemplary nucleotide incorporating enzymes include, e.g., polymerases, terminal transferases, reverse transcriptases, telomerases, polynucleotide phosphorylases, and the like.

[0072] An "**oligonucleotide**" refers to a nucleic acid that includes at least two nucleic acid monomer units (e.g., nucleotides), typically more than three monomer units, and more typically greater than ten monomer units. The exact size of an oligonucleotide generally depends on various factors, including the ultimate function or use of the oligonucleotide. Oligonucleotides are optionally prepared by any suitable method, including, but not limited to, isolation of an existing or natural sequence, DNA replication or amplification, reverse transcription, cloning and restriction digestion of appropriate sequences, or direct chemical synthesis by a method such as the phosphotriester method of Narang et al. (1979) Meth. Enzymol. 68:90-99; the phosphodiester method of Brown et al. (1979) Meth. Enzymol. 68:109-151; the diethylphosphoramidite method of Beaucage et al. (1981) Tetrahedron Lett. 22:1859-1862; the triester method of Matteucci et al. (1981) J. Am. Chem. Soc. 103:3185-3191; automated synthesis methods; or the solid support method of U.S. Pat. No. 4,458,066,

or other methods known in the art. All of these references are incorporated by reference.

[0073] The term "**probe nucleic acid**" or "**probe**" refers to a labeled or unlabeled oligonucleotide capable of selectively hybridizing to a target nucleic acid under suitable conditions. Typically, a probe is sufficiently complementary to a specific target sequence contained in a SARS-CoV nucleic acid sample to form a stable hybridization duplex with the target sequence under a selected hybridization condition, such as, but not limited to, a stringent hybridization condition. A hybridization assay carried out using the probe under sufficiently stringent hybridization conditions permits the selective detection of a specific target sequence. The term "**hybridizing region**" refers to that region of a nucleic acid that is exactly or substantially complementary to, and therefore hybridizes to, the target sequence. For use in a hybridization assay for the discrimination of single nucleotide differences in sequence, the hybridizing region is typically from about 8 to about 100 nucleotides in length. Although the hybridizing region generally refers to the entire oligonucleotide, the probe may include additional nucleotide sequences that function, for example, as linker binding sites to provide a site for attaching the probe sequence to a solid support or the like. In certain embodiments, a probe of the invention comprises one or more labels (e.g., a reporter dye, a quencher moiety, etc.), such as a 5'-nuclease probe, a FRET probe, a molecular beacon, or the like, which can also be utilized to detect hybridization between the probe and target nucleic acids in a sample. In some embodiments, the hybridizing region of the probe is completely complementary to the target sequence. However, in general, complete complementarity is not necessary; stable duplexes may contain mismatched bases or unmatched bases. Modification of the stringent conditions may be necessary to permit a stable hybridization duplex with one or more base pair mismatches or unmatched bases. Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2001), which is incorporated by reference, provides guidance for suitable modification. Stability of the target/probe duplex depends on a number of variables including length of the oligonucleotide, base composition and sequence of the oligonucleotide, temperature, and ionic conditions. One of skill in the art will recognize that, in general, the exact complement of a given probe is similarly useful as a probe. Exemplary probes of the

invention that bind to SARS-CoV nucleic acids include oligonucleotides that comprise sequences selected from the group consisting of: SEQ ID NOS: 1-12 and 15-24, a substantially identical variant thereof wherein the variant has at least 90% sequence identity to one of SEQ ID NOS: 1-12 and 15-24, and complements of SEQ ID NOS: 1-12 and 15-24 and the variant. One of skill in the art will also recognize that, in certain embodiments, probe nucleic acids can also be used as primer nucleic acids.

[0074] A "**primer nucleic acid**" or "**primer**" is a nucleic acid that can hybridize to a template nucleic acid (e.g., a SARS-CoV nucleic acid) and permit chain extension or elongation using, e.g., a nucleotide incorporating biocatalyst, such as a polymerase under appropriate reaction conditions. A primer nucleic acid is typically a natural or synthetic oligonucleotide (e.g., a single-stranded oligodeoxyribonucleotide, etc.). Although other primer nucleic acid lengths are optionally utilized, they typically comprise hybridizing regions that range from about 8 to about 100 nucleotides in length. Short primer nucleic acids generally utilize cooler temperatures to form sufficiently stable hybrid complexes with template SARS-CoV nucleic acid. A primer nucleic acid that is at least partially complementary to a subsequence of a template SARS-CoV nucleic acid is typically sufficient to hybridize with the template for extension to occur. A primer nucleic acid can be labeled, if desired, by incorporating a label detectable by, e.g., spectroscopic, photochemical, biochemical, immunochemical, chemical, or other techniques. To illustrate, useful labels include radioisotopes, fluorescent dyes, electron-dense reagents, enzymes (as commonly used in ELISAs), biotin, or haptens and proteins for which antisera or monoclonal antibodies are available. Many of these and other labels are described further herein and/or are otherwise known in the art. Exemplary primer nucleic acids of the invention that bind to SARS-CoV nucleic acids include oligonucleotides that comprise sequences selected from the group consisting of: SEQ ID NOS: 1-12 and 15-24, a substantially identical variant thereof wherein the variant has at least 90% sequence identity to one of SEQ ID NOS: 1-12 and 15-24, and complements of SEQ ID NOS: 1-12 and 15-24 and the variant. One of skill in the art will recognize that, in certain embodiments, primer nucleic acids can also be used as probe nucleic acids.

[0075] A "**quencher moiety**" or "**quencher**" refers to a moiety that reduces and/or is capable of reducing the detectable emission of radiation, e.g., fluorescent or

luminescent radiation, from a source that would otherwise have emitted this radiation. A quencher typically reduces the detectable radiation emitted by the source by at least 50%, typically by at least 80%, and more typically by at least 90%. Exemplary quenchers are provided in, e.g., U.S. Pat. No. 6,465,175, entitled "OLIGONUCLEOTIDE PROBES BEARING QUENCHABLE FLUORESCENT LABELS, AND METHODS OF USE THEREOF," which issued October 15, 2002 to Horn et al., which is incorporated by reference.

[0076] The term "**sample**" refers to any substance containing or presumed to contain one or more SARS-CoV nucleic acids including, but not limited to, clinical samples (e.g., tissue or fluid isolated from one or more subjects or individuals), in vitro cell culture constituents, environmental samples, and the like. Exemplary samples include blood, plasma, serum, feces, bronchoalveolar lavage, nasal pharyngeal swabs and tissues, urine, synovial fluid, seminal fluid, seminal plasma, prostatic fluid, vaginal fluid, cervical fluid, uterine fluid, cervical scrapings, amniotic fluid, anal scrapings, mucus, sputum, tissue, and the like.

[0077] The phrase "**sample derived from a subject**" refers to a sample obtained from the subject, whether or not that sample undergoes one or more processing steps (e.g., cell lysis, debris removal, stabilization, etc.) prior to analysis. To illustrate, samples can be derived from subjects by scraping, venipuncture, swabbing, biopsy, or other techniques known in the art.

[0078] The term "**severe acute respiratory syndrome coronavirus**" or "**SARS-CoV**" refers to a positive-strand RNA-enveloped virus of the family *Coronaviridae* and the order *Nidovirales*, which causes the severe acute respiratory syndrome (SARS) in subjects infected with the virus. *See, e.g.*, Kiazek et al. (2003) "A novel coronavirus associated with severe acute respiratory syndrome," N. Engl. J. Med. 348:1953–1966, Drosten et al. (2003) "Identification of a novel coronavirus in patients with severe acute respiratory syndrome," N. Engl. J. Med. 348:1967–1976, Marra et al. (2003) "The genome sequence of the SARS-associated coronavirus," Science 300:1399–1404, Ruan et al. (2003) "Comparative full-length genome sequence analysis of 14 SARS coronavirus isolates and common mutations associated with putative origins of infection," Lancet 361:1779–1785, and Cavanagh (2003) "*Nidovirales*: a new order comprising *Coronaviridae* and *Arteriviridae*," Arch. Virol. 14:629–633, which

are each incorporated by reference. Exemplary GenBank® accession numbers for complete sequences of the SARS-CoV genome are NC_004718, AY283794, AY278489, AY278741, AY559097, AY559096, AY559095, AY559094, AY559093, AY559092, AY559091, AY559090, AY559089, AY559088, AY559087, AY559086, AY559085, AY559084, AY559083, AY559082, AY559081, AY274119, AY323977, AY291315, and the like. To further illustrate, Figure 1 provides the nucleotide sequence of the SARS-CoV genome (Urbani strain) as determined by the Centers for Disease Control.

[0079] The term "**severe acute respiratory syndrome coronavirus nucleic acid**" or "**SARS-CoV nucleic acid**" refers to a nucleic acid that is derived or isolated from a SARS-CoV and/or an amplicon thereof.

[0080] The term "**selectively bind**" or "**selective binding**" in the context of nucleic acid detection reagents refers to a nucleic acid detection reagent that binds to SARS-CoV nucleic acids to a greater extent than the nucleic acid detection reagent binds, under the same hybridization conditions, to non-target nucleic acids.

[0081] The term "**selectively detect**" refers to the ability to detect a SARS-CoV nucleic acid to a greater extent than nucleic acids from other organisms.

[0082] "**Selectively hybridizing**" or "**selective hybridization**" occurs when a nucleic acid sequence hybridizes to a specified nucleic acid target sequence to a detectably greater degree than its hybridization to non-target nucleic acid sequences. Selectively hybridizing sequences have at least 50%, or 60%, or 70%, or 80%, or 90% sequence identity or more, e.g., typically 95-100% sequence identity (i.e., complementarity) with each other.

[0083] A "**sequence**" of a nucleic acid refers to the order and identity of nucleotides in the nucleic acid. A sequence is typically read in the 5' to 3' direction.

[0084] A "**sequence specific antibody**" refers to an antibody that detectably binds to SARS-CoV nucleic acids.

[0085] A "**sequencing reaction**" refers to a reaction that includes, e.g., the use of terminator nucleotides and which is designed to elucidate the sequence of nucleotides in a given nucleic acid.

[0086] A "**set**" refers to a collection of at least two things. For example, a set may include 2, 3, 4, 5, 10, 20, 50, 100, 1,000 or other number of molecule or sequence types. For example, certain aspects of the invention include reaction mixtures having sets of amplicons. A "**subset**" refers to any portion of a set.

[0087] A "**solid support**" refers to a solid material that can be derivatized with, or otherwise attached to, a chemical moiety, such as an oligonucleotide or the like. Exemplary solid supports include plates, beads, microbeads, tubes, fibers, whiskers, combs, hybridization chips (including microarray substrates, such as those used in GeneChip® probe arrays (Affymetrix, Inc., Santa Clara, CA, USA) and the like), membranes, single crystals, ceramic layers, self-assembling monolayers, and the like.

[0088] An oligonucleotide is "**specific**" for a target sequence if the number of mismatches present between the oligonucleotide and the target sequence is less than the number of mismatches present between the oligonucleotide and non-target sequences that might be present in a sample. Hybridization conditions can be chosen under which stable duplexes are formed only if the number of mismatches present is no more than the number of mismatches present between the oligonucleotide and the target sequence. Under such conditions, the target-specific oligonucleotide can form a stable duplex only with a target sequence. Thus, the use of target-specific primers under suitably stringent amplification conditions enables the specific amplification of those sequences, which contain the target primer binding sites. Similarly, the use of target-specific probes under suitably stringent hybridization conditions enables the detection of a specific target sequence.

[0089] A test nucleic acid is said to "**specifically hybridize**" to an oligonucleotide when it hybridizes at least one-half as well to the oligonucleotide as to the perfectly matched complementary target, i.e., with a signal to noise ratio at least one-half as high as hybridization of the oligonucleotide to the target under conditions in which the perfectly matched oligonucleotide binds to the perfectly matched complementary target with a signal to noise ratio that is at least about 5x-10x as high as that observed for hybridization to non-target nucleic acids present in a sample.

[0090] A "**subject**" refers to an organism. Typically, the organism is a mammalian organism, particularly a human organism. In certain embodiments, for example, a subject is a patient suspected of having a SARS-CoV infection.

[0091] A "**subsequence**" or "**segment**" refers to any portion of an entire nucleic acid sequence.

[0092] A "**substantially identical variant**" in the context of nucleic acids or polypeptides, refers to two or more sequences that have at least 85%, typically at least 90%, more typically at least 95% nucleotide or sequence identity to one another when compared and aligned for maximum correspondence, as measured using, e.g., a sequence comparison algorithm or by visual inspection. The substantial identity generally exists over a region of the sequences that is at least about 15 nucleotides or amino acids in length, more typically over a region that is at least about 20 nucleotides or amino acids in length, and even more typically the sequences are substantially identical over a region of at least about 25 nucleotides or amino acids in length. In some embodiments, for example, the sequences are substantially identical over the entire length of the nucleic acids or polypeptides being compared.

[0093] The term "**substitution**" in the context of a nucleic acid sequence refers to an alteration in which at least one nucleotide of the nucleic acid sequence is replaced by a different nucleotide.

[0094] The terms "**target sequence**," "**target region**," and "**target nucleic acid**" refer to a region of a nucleic acid, which is to be amplified, detected, or otherwise analyzed.

[0095] A "**terminator nucleotide**" refers to a nucleotide, which upon incorporation into a nucleic acid prevents further extension of the nucleic acid, e.g., by at least one nucleotide incorporating biocatalyst.

[0096] A "**thermostable enzyme**" refers to an enzyme that is stable to heat, is heat resistant and retains sufficient catalytic activity when subjected to elevated temperatures for selected periods of time. For example, a thermostable polymerase retains sufficient activity to effect subsequent primer extension reactions when subjected to elevated temperatures for the time necessary to effect denaturation of double-stranded nucleic acids. Heating conditions necessary for nucleic acid

denaturation are well known in the art and are exemplified in U.S. Pat. Nos. 4,683,202 and 4,683,195, which are both incorporated by reference. As used herein, a thermostable polymerase is typically suitable for use in a temperature cycling reaction such as the polymerase chain reaction ("PCR"). For a thermostable polymerase, enzymatic activity refers to the catalysis of the combination of the nucleotides in the proper manner to form primer extension products that are complementary to a template nucleic acid (e.g., SARS-CoV nucleic acids).

II. OVERVIEW

[0097] The Centers for Disease Control and Prevention (CDC) has sequenced the genome for a coronavirus believed to be responsible for the global epidemic of severe acute respiratory syndrome or SARS. Figure 1 shows this nucleotide sequence of the SARS-CoV genome, which corresponds to GenBank® accession number AY278741. *See also,* Rota et al. (2003) "Characterization of a novel coronavirus associated with severe acute respiratory syndrome," Science 300(5624):1394-1399, which is incorporated by reference. The sequence data confirm that the SARS coronavirus is a previously unrecognized coronavirus. All of the sequence, except for the leader sequence, was derived directly from viral RNA. The genome of the SARS coronavirus sequenced by the CDC is 29,727 nucleotides in length and the genome organization is similar to that of other coronaviruses. *See, e.g.,* Drosten et al. (2003) "Identification of a novel coronavirus in patients with severe acute respiratory syndrome," N. Engl. J. Med. 348:1967–1976, which is incorporated by reference. Open reading frames corresponding to the polymerase protein (polymerase 1a, 1b), spike protein (S), small membrane protein (E), membrane protein (M) and nucleocapsid protein (N) have been identified.

[0098] The invention relates to the selective detection of SARS-CoV. In particular, based on new detection strategies utilizing selected target regions of the SARS-CoV genome, SARS-CoV infections can be diagnosed using the methods and reagents described herein. In certain embodiments, for example, certain nucleic acid detection reagents described herein target a region of the SARS-CoV genome that encodes an RNA-dependent RNA polymerase (RdRp), which has been shown to be stable against mutations compared to other regions of the SARS-CoV genome. The

nucleic acid detection reagents described herein generally detectably bind, under selected assay conditions, to nucleotide sequences that are present in SARS-CoV, but which are not present in other species, thereby minimizing the occurrence of, e.g., false positives. The detection of SARS-CoV nucleic acids with certain nucleic acid detection reagents of the invention is illustrated in, for example, in the examples provided below. Many other features of the invention are also described herein.

[0099] To further illustrate, certain methods of the invention include contacting or incubating nucleic acid detection reagents with nucleic acids in or from samples derived from subjects (e.g., human patients suspected of having SARS-CoV infections, etc.). In certain embodiments, target regions of the nucleic acids in the samples are amplified prior to or simultaneously with being contacted with the nucleic acid detection reagents. These methods also include monitoring (e.g., at a single time point, at multiple discrete time points, continuously over a selected time period, etc.) binding between the nucleic acids and/or amplicons, and the nucleic acid detection reagents to determine whether the SARS-CoV is present in the samples, e.g., to diagnose patients from which the samples were derived, to monitor courses of treatment for patients diagnosed with SARS infections, and/or the like.

[0100] Other methods of the invention include contacting or incubating nucleic acids from samples with at least one pair of primer nucleic acids that include at least one nucleic acid having a sequence selected from the group consisting of: SEQ ID NOS: 1-12 and 15-24, a substantially identical variant thereof in which the variant has at least 90% sequence identity to one of SEQ ID NOS: 1-12 and 15-24, and complements of SEQ ID NOS: 1-12 and 15-24 and the variant, in nucleic acid amplification reactions. In addition, these methods also include detecting amplicons during or after the amplification reactions are performed to detect whether the SARS-CoV is present in the samples. These methods are also optionally repeated at selected time points.

[0101] In addition to compositions and reaction mixtures, the invention also relates to kits and systems for detecting SARS-CoV, and to related computers and computer readable media.

III. NUCLEIC ACID DETECTION REAGENTS

[0102] The nucleic acid detection reagents of the invention include various embodiments, including probe nucleic acids, primer nucleic acids, and sequence specific antibodies. In some embodiments, for example, certain nucleic acid detection reagents described herein target a segment of the SARS-CoV genome that encodes an RdRp. This region of the SARS-CoV genome is also referred to in, e.g., Ng et. (2004) "Detection of Severe Acute Respiratory Syndrome Coronavirus in Blood of Infected Patients" J. Clin. Microbiol. 42:347-350, which is incorporated by reference.

[0103] Exemplary nucleic acid detection reagents that target SARS-CoV nucleic acids include oligonucleotides comprising sequences selected from SEQ ID NOS: 1-12 and 15-24 or complements thereof. SEQ ID NOS: 1-12 and 15-24 are shown in Table I.

TABLE I

SEQ ID NO: 1	5'- TGATGGTTGTGTTCCACTCAACATC -3'
SEQ ID NO: 2	5'- ACACTGGTACAGGACAGGCAATTAC -3'
SEQ ID NO: 3	5'- GGTGTAAGTGCAGCCCGTCTTACAC -3'
SEQ ID NO: 4	5'- CGAATTTTGCTCACAGCATACAATG -3'
SEQ ID NO: 5	5'- ATTGGAGAGTACACCTTTGAAAAAG -3'
SEQ ID NO: 6	5'- GCTAGACTTCGTGCAAAACACTACG -3'
SEQ ID NO: 7	5'- TGTGGCTAGTTGTGATGCTATCATG -3'
SEQ ID NO: 8	5'- GCTACACATCACGATAAATTCACTG -3'
SEQ ID NO: 9	5'- CACTCAAATCTGCTACGTGTATTAC -3'
SEQ ID NO: 10	5'- CTCGCTATGGATGAATTCATACAGC -3'
SEQ ID NO: 11	5'- GGTTGGGATTATCCAAAATGTGA -3'
SEQ ID NO: 12	5'- GGCATCATCAGAAAGAATCATCAT -3'
SEQ ID NO: 15	5'- ACTATATGTTAAACCAGGTGG -3'
SEQ ID NO: 16	5'- ATTTACATTGGCTGTAACAGC -3'
SEQ ID NO: 17	5'- AGCTAACGAGTGTGCGCAAGTATTAAGTGAGATG -3'
SEQ ID NO: 18	5'- CCTCTCTTGTTCTTGCTCGCAAAC -3'
SEQ ID NO: 19	5'- AGAACAAGAGAGGCCATTATCCTAAG -3'
SEQ ID NO: 20	5'- TTAACATATAGTGAGCCGCCACAC -3'
SEQ ID NO: 21	5'- AGAGCCATGCCTAACAT -3'
SEQ ID NO: 22	5'- GGTTGGGATTATCCAAAATGTGAC -3'
SEQ ID NO: 23	5'- GGTTGGGATTATCCAAAATGTGA -3'
SEQ ID NO: 24	5'- GTGTGGCGGCTCACTATATGTTA -3'

[0104] To further illustrate, Figure 2 shows alignments of nucleotide sequences from various SARS-CoV isolates and from certain non-target organisms relative to one another. As shown, Group 1 shows the alignment of non-target nucleotide sequences

from the Human Coronavirus (HCoV) 229E (Accession No. NC_002645) and the Porcine epidemic diarrhea virus (PEDV) (Accession No. NC_003436) with target nucleotide sequences from three different SARS-CoV isolates, namely, Toronto (Accession No. NC_004718), Singapore (Accession No. AY283794), and Guangzhou (Accession No. AY278489). As shown, the positions of oligonucleotides with sequences corresponding to SEQ ID NOS: 23 and 24 are underlined. Group 2 shows the alignment of non-target nucleotide sequences from the Bovine Coronavirus (BCoV) (Accession No. NC_003045) and the Murine hepatitis virus (MHV) (Accession No. NC_001846) with the target nucleotide sequences from the different SARS-CoV isolates shown in Group 1. The positions of oligonucleotides with sequences corresponding to SEQ ID NOS: 23 and 24 are also underlined in the alignment of Group 2. Group 3 illustrates the alignment of a non-target nucleotide sequence from the Avian Infectious Bronchitis Virus (IBV) (Accession No. NC_001451) with the target nucleotide sequences from the different SARS-CoV isolates shown in Group 1. The positions of oligonucleotides with sequences corresponding to SEQ ID NOS: 23 and 24 are also underlined in the alignment of Group 3.

[0105] As mentioned above, nucleic acid detection reagents comprise oligonucleotides (e.g., probe nucleic acids, primer nucleic acids, etc.) in certain embodiments of the invention. Although other lengths are optionally utilized, oligonucleotides generally comprise sequences that are typically between about 8 and about 100 nucleotides in length, more typically between about 10 and about 75 nucleotides in length, still more typically between about 12 and about 50 nucleotides in length, and even more typically between about 15 and about 35 nucleotides in length (e.g., about 20, about 25, or about 30 nucleotides in length). Methods of preparing oligonucleotides, such as nucleic acid synthesis, are described further below.

[0106] Various approaches can be utilized by one of skill in the art to design oligonucleotides (e.g., substantially identical variants of nucleic acids having sequences selected from SEQ ID NOS: 1-12 and 15-24 or complements thereof) that selectively bind to SARS-CoV nucleic acids. To illustrate, the DNAstar software package available from DNASTAR, Inc. (Madison, WI, USA) can be used for sequence alignments. For example, nucleic acid sequences for the SARS-CoV genome and non-

target sequences can be uploaded into DNAstar EditSeq program as individual files. To further illustrate, pairs of sequence files can be opened in the DNAstar MegAlign sequence alignment program and the Clustal W method of alignment can be applied. The parameters used for Clustal W alignments are optionally the default settings in the software. MegAlign typically does not provide a summary of the percent identity between two sequences. This is generally calculated manually. From the alignments, regions having, e.g., less than 85% identity with one another are typically identified and oligonucleotide sequences in these regions can be selected. Many other sequence alignment algorithms and software packages are also optionally utilized. Sequence alignment algorithms are also described in, e.g., Mount, Bioinformatics: Sequence and Genome Analysis, Cold Spring Harbor Laboratory Press (2001), and Durbin et al., Biological Sequence Analysis: Probabilistic Models of Proteins and Nucleic Acids, Cambridge University Press (1998), which are both incorporated by reference.

[0107] To further illustrate, optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman (1981) Adv. Appl. Math. 2:482, by the homology alignment algorithm of Needleman & Wunsch (1970) J. Mol. Biol. 48:443, by the search for similarity method of Pearson & Lipman (1988) Proc. Nat'l. Acad. Sci. USA 85:2444, which are each incorporated by reference, and by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (Madison, WI), or by even by visual inspection.

[0108] Another example algorithm that is suitable for determining percent sequence identity is the BLAST algorithm, which is described in, e.g., Altschul et al. (1990) J. Mol. Biol. 215:403-410, which is incorporated by reference. Software for performing versions of BLAST analyses is publicly available through the National Center for Biotechnology Information on the world wide web at ncbi.nlm.nih.gov/ as of April 20, 2004. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., *supra*). These initial neighborhood word hits act as

seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see, Henikoff & Henikoff (1989) Proc. Natl. Acad. Sci. USA 89:10915, which is incorporated by reference).

[0109] In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul (1993) Proc. Nat'l. Acad. Sci. USA 90:5873-5787, which is incorporated by reference). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence (and, therefore, homologous) if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, or less than about 0.01, and or even less than about 0.001.

[0110] An additional example of a useful sequence alignment algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle (1987) J. Mol. Evol. 35:351-360,

which is incorporated by reference. The method used is similar to the method described by Higgins & Sharp (1989) CABIOS 5:151-153, which is incorporated by reference. The program can align, e.g., up to 300 sequences of a maximum length of 5,000 letters. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster can then be aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences can be aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program can also be used to plot a dendogram or tree representation of clustering relationships. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison.

[0111] The probes and primers of the invention optionally include one or more labels, which are described further below. In addition, probes and primers optionally include various other modifications, such as modified nucleotides that alter hybridization melting temperatures, restriction site linkers to facilitate amplicon cloning, modifier groups that increase the specificity of nucleic acid amplification reactions, and/or the like. For example, certain modified nucleotides that increase nucleic acid hybridization melting temperatures are optionally included to permit the use of smaller probes and primers, such as those including between about 8 and about 14 nucleotides. Examples of these modified oligonucleotides include those having one or more LNA™ monomers. Nucleotide analogs such as these are described further in, e.g., U.S. Pat. No. 6,639,059, entitled "SYNTHESIS OF [2.2.1]BICYCLO NUCLEOSIDES," issued October 28, 2003 to Kochkine et al., U.S. Pat. No. 6,303,315, entitled "ONE STEP SAMPLE PREPARATION AND DETECTION OF NUCLEIC ACIDS IN COMPLEX BIOLOGICAL SAMPLES," issued October 16, 2001 to Skouv, and U.S. Pat. Application Pub. No. 2003/0092905, entitled "SYNTHESIS OF [2.2.1]BICYCLO NUCLEOSIDES," by Kochkine et al. that published May 15, 2003, which are each incorporated by reference. Oligonucleotides comprising LNA™ monomers are commercially available through, e.g., Exiqon A/S (Vedbæk, DK). Additional probe and primer modifications are referred to herein, including in the definitions provided above.

[0112] In certain embodiments, the nucleic acid detection reagents utilized as described herein are sequence specific antibodies that target SARS-CoV nucleic acids. Antibodies suitable for use in these embodiments of invention may be prepared by conventional methodology and/or by genetic engineering. Antibody fragments may be genetically engineered, e.g., from the variable regions of the light and/or heavy chains (V_H and V_L), including the hypervariable regions, or from both the V_H and V_L regions. For example, the term "antibodies" as used herein includes polyclonal and monoclonal antibodies and biologically active fragments thereof including among other possibilities "univalent" antibodies (Glennie et al. (1982) Nature 295:712); Fab proteins including Fab' and $F(ab')_2$ fragments whether covalently or non-covalently aggregated; light or heavy chains alone, typically variable heavy and light chain regions (V_H and V_L regions), and more typically including the hypervariable regions (otherwise known as the complementarity determining regions (CDRs) of the V_H and V_L regions); F_c proteins; "hybrid" antibodies capable of binding more than one antigen; constant-variable region chimeras; "composite" immunoglobulins with heavy and light chains of different origins; "altered" antibodies with improved specificity and other characteristics as prepared by standard recombinant techniques, by mutagenic techniques, or other directed evolutionary techniques known in the art.

[0113] The sequence specific antibodies utilized as described herein may be labeled or unlabeled. Suitable labels include, e.g., radionuclides, enzymes, coenzymes, fluorescent dyes, chemiluminescent dyes, chromogens, enzyme substrates or co-factors, enzyme inhibitors, free radicals, and the like. Such labeled reagents may be used in a variety of well known assays, such as radioimmunoassays, enzyme immunoassays, e.g., ELISA, fluorescent immunoassays, and the like. See, e.g., U.S. Pat. Nos. 3,766,162; 3,791,932; 3,817,837; and 4,233,402, which are each incorporated by reference. Additional labels are described further herein.

[0114] In some embodiments, polypeptides, such the RdRp referred to above are targeted for detection. Many techniques for detecting proteins are known in the art. For example, various electrophoretic assays (e.g., SDS-PAGE or the like), immunoassays, mass spectrometric assays (e.g., matrix assisted laser desorption/ionization (MALDI)-based analyses, surface enhanced laser

desorption/ionization (SELDI)-based assays, etc.), and/or other approaches can be used to detect proteins encoded by SARS-CoV nucleic acids. Many of these and other suitable protein detection methods are described in the references cited herein.

[0115] In practicing the present invention, many conventional techniques in molecular biology and recombinant DNA are optionally used. These techniques are well known and are explained in, for example, Current Protocols in Molecular Biology, Volumes I, II, and III, 1997 (F. M. Ausubel ed.); Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001; Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology volume 152 Academic Press, Inc., San Diego, CA (Berger), DNA Cloning: A Practical Approach, Volumes I and II, 1985 (D. N. Glover ed.); Oligonucleotide Synthesis, 1984 (M. L. Gait ed.); Nucleic Acid Hybridization, 1985, (Hames and Higgins); Transcription and Translation, 1984 (Hames and Higgins eds.); Animal Cell Culture, 1986 (Freshney ed.); Immobilized Cells and Enzymes, 1986 (IRL Press); Perbal, 1984, A Practical Guide to Molecular Cloning; the series, Methods in Enzymology (Academic Press, Inc.); Gene Transfer Vectors for Mammalian Cells, 1987 (J. H. Miller and M. P. Calos eds., Cold Spring Harbor Laboratory); Methods in Enzymology Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively), all of which are incorporated by reference.

IV. SEQUENCE VARIATIONS

[0116] Numerous nucleic acid and polypeptide sequences are within the scope of the present invention, whether as target sequences or the agents used to detect those target sequences.

SILENT VARIATIONS

[0117] It will be appreciated by those skilled in the art that due to the degeneracy of the genetic code, a multitude of SARS-CoV nucleic acids sequences encoding polypeptides may be produced, some of which may bear minimal sequence homology to the nucleic acid sequences explicitly disclosed herein. For instance, inspection of the codon table (Table II) shows that codons AGA, AGG, CGA, CGC, CGG, and CGU all encode the amino acid arginine. Thus, at every position in the

nucleic acids of the invention where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described above without altering the encoded polypeptide. It is understood that U in an RNA sequence corresponds to T in a DNA sequence.

TABLE II
Codon Table

Amino acids			Codon					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

[0118] Such "silent variations" are one species of "conservatively modified variations", discussed below. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified by standard techniques to encode a functionally identical polypeptide. Accordingly, each silent variation of a nucleic acid, which encodes a polypeptide is implicit in any described sequence. For example, the invention provides each and every possible variation of nucleic acid sequence encoding an RdRp of a SARS-CoV that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code (e.g., as set forth in Table

II) as applied to the nucleic acid sequences encoding, e.g., an RdRp of a SARS-CoV. All such variations of every nucleic acid herein are specifically provided and described by consideration of the sequence in combination with the genetic code.

CONSERVATIVE VARIATIONS

[0119] "Conservatively modified variations" or, simply, "conservative variations" of a particular nucleic acid sequence refers to those nucleic acids, which encode identical or essentially identical amino acid sequences, or, where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. One of skill will recognize that individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 4%, 2% or 1%) in an encoded sequence are "conservatively modified variations" where the alterations result in the deletion of an amino acid, addition of an amino acid, or substitution of an amino acid with a chemically similar amino acid.

[0120] Conservative substitution tables providing functionally similar amino acids are well known in the art. Table III sets forth six groups, which contain amino acids that are "conservative substitutions" for one another.

TABLE III
Conservative Substitution Groups

1	Alanine (A)	Serine (S)	Threonine (T)	
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Lysine (K)		
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

[0121] Thus, "conservatively substituted variations" of, e.g., an RdRp of a SARS-CoV include substitutions of a small percentage, typically less than 5%, more typically less than 2% or 1%, of the amino acids of the polypeptide sequence, with a conservatively selected amino acid of the same conservative substitution group.

[0122] The addition of sequences that do not alter the encoded activity of a nucleic acid molecule, such as the addition of a non-functional sequence, is a conservative variation of the basic nucleic acid.

[0123] One of skill will appreciate that many conservative variations of the nucleic acids described herein yield a functionally identical nucleic acid. For example, as discussed above, owing to the degeneracy of the genetic code, "silent substitutions" (*i.e.*, substitutions in a nucleic acid sequence which do not result in an alteration in an encoded polypeptide) are an implied feature of *every* nucleic acid sequence, which encodes an amino acid. Similarly, "conservative amino acid substitutions," in one or a few amino acids in an amino acid sequence are substituted with different amino acids with highly similar properties, are also readily identified as being highly similar to a disclosed construct. Such conservative variations of each disclosed sequence are a feature of the present invention.

V. OLIGONUCLEOTIDE SYNTHESIS

[0124] The oligonucleotides of the invention are optionally prepared using essentially any technique known in the art. In certain embodiments, for example, the oligonucleotides described herein are synthesized chemically using essentially any nucleic acid synthesis method, including, e.g., according to the solid phase phosphoramidite triester method described by Beaucage and Caruthers (1981), Tetrahedron Letts. 22(20):1859-1862, which is incorporated by reference, or another synthesis technique known in the art, *e.g.*, using an automated synthesizer, as described in Needham-VanDevanter et al. (1984) Nucleic Acids Res. 12:6159-6168, which is incorporated by reference. A wide variety of equipment is commercially available for automated oligonucleotide synthesis. Multi-nucleotide synthesis approaches (e.g., tri-nucleotide synthesis, etc.) are also optionally utilized. Moreover, the primer nucleic acids described herein optionally include various modifications. In certain embodiments, for example, primers include restriction site linkers, e.g., to facilitate subsequent amplicon cloning or the like. To further illustrate, primers are also optionally modified to improve the specificity of amplification reactions as described in, e.g., U.S. Pat. No. 6,001,611, entitled "MODIFIED NUCLEIC ACID AMPLIFICATION PRIMERS," issued December 14, 1999 to Will, which is

incorporated by reference. Primers and probes can also be synthesized with various other modifications as described herein or as otherwise known in the art.

[0125] Essentially any label is optionally utilized to label the nucleic acid detection reagents of the invention. In some embodiments, for example, the label comprises a fluorescent dye (e.g., a rhodamine dye (e.g., R6G, R110, TAMRA, ROX, etc.), a fluorescein dye (e.g., JOE, VIC, TET, HEX, FAM, etc.), a halofluorescein dye, a cyanine dye (e.g., CY3, CY3.5, CY5, CY5.5, etc.), a BODIPY® dye (e.g., FL, 530/550, TR, TMR, etc.), an ALEXA FLUOR® dye (e.g., 488, 532, 546, 568, 594, 555, 653, 647, 660, 680, etc.), a dichlororhodamine dye, an energy transfer dye (e.g., BIGDYE™ v 1 dyes, BIGDYE™ v 2 dyes, BIGDYE™ v 3 dyes, etc.), Lucifer dyes (e.g., Lucifer yellow, etc.), CASCADE BLUE®, Oregon Green, and the like. Additional examples of fluorescent dyes are provided in, e.g., Haugland, Molecular Probes Handbook of Fluorescent Probes and Research Products, Ninth Ed. (2003) and the updates thereto, which are each incorporated by reference. Fluorescent dyes are generally readily available from various commercial suppliers including, e.g., Molecular Probes, Inc. (Eugene, OR), Amersham Biosciences Corp. (Piscataway, NJ), Applied Biosystems (Foster City, CA), etc. Other labels include, e.g., biotin, weakly fluorescent labels (Yin et al. (2003) Appl Environ Microbiol. 69(7):3938, Babendure et al. (2003) Anal. Biochem. 317(1):1, and Jankowiak et al. (2003) Chem Res Toxicol. 16(3):304), non-fluorescent labels, colorimetric labels, chemiluminescent labels (Wilson et al. (2003) Analyst. 128(5):480 and Roda et al. (2003) Luminescence 18(2):72), Raman labels, electrochemical labels, bioluminescent labels (Kitayama et al. (2003) Photochem Photobiol. 77(3):333, Arakawa et al. (2003) Anal. Biochem. 314(2):206, and Maeda (2003) J. Pharm. Biomed. Anal. 30(6):1725), and an alpha-methyl-PEG labeling reagent as described in, e.g., U.S. Provisional Patent Application No. 60/428,484, filed on Nov. 22, 2002, which references are each incorporated by reference. Nucleic acid labeling is also described further below.

[0126] In addition, essentially any nucleic acid (and virtually any labeled nucleic acid, whether standard or non-standard) can be custom or standard ordered from any of a variety of commercial sources, such as The Midland Certified Reagent

Company, The Great American Gene Company, ExpressGen Inc., Operon Technologies Inc., Proligo LLC, and many others.

VI. SAMPLE PREPARATION AND NUCLEIC ACID AMPLIFICATION

[0127] Samples are generally derived or isolated from subjects, typically mammalian subjects, more typically human subjects, suspected of having a SARS-CoV infection. Exemplary samples or specimens include blood, plasma, serum, feces, bronchoalveolar lavage, nasal pharyngeal swabs and tissues, urine, synovial fluid, seminal fluid, seminal plasma, prostatic fluid, vaginal fluid, cervical fluid, uterine fluid, cervical scrapings, amniotic fluid, anal scrapings, mucus, sputum, tissue, and the like. Essentially any technique for acquiring these samples is optionally utilized including, e.g., scraping, venipuncture, swabbing, biopsy, or other techniques known in the art. Methods of storing specimens, culturing cells, isolating and preparing nucleic acids from these sources are generally known in the art and many of these are described further in the references and/or examples provided herein. A detailed description of recommended facilities, practices, and protective equipment for the various laboratory biosafety levels (BSLs) may also be found in the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories manual (BMBL), which is available on the world wide web at cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm as of April 20, 2004.

[0128] To illustrate, blood and urine specimens may be handled using Standard Precautions (previously Universal Precautions) in BSL-2 laboratories. Laboratory workers should wear protective equipment, including disposable gloves, laboratory coats, eye protection and a surgical mask, or face shield to provide a barrier to mucosal surface exposure. Careful attention should be given to hand hygiene after removal of gloves and especially before touching the eyes or mucosal surfaces.

[0129] Any procedure with the potential to generate fine particulate aerosols (e.g. vortexing or sonication of specimens in an open tube) should be performed in a biological safety cabinet (BSC). The use of sealed centrifuge rotors or sample cups, if available, should be employed for centrifugation. Ideally, these rotors or cups should be unloaded in a BSC.

[0130] Procedures performed outside of a BSC should be performed in a manner that minimizes the risk of exposure to an inadvertent sample release.

[0131] Work surfaces and equipment should be decontaminated after specimens are processed. Standard decontamination agents that are effective against lipid-enveloped viruses should be sufficient.

[0132] If the safety equipment described above is not available, administrative measures and/or additional personal protective equipment may be employed to reduce risk. This should be done in the context of a careful risk assessment by the laboratory safety officer. For example, the workflow of the laboratory may be adjusted so that a minimum number of workers are present during centrifugation.

[0133] Consideration may be given to implementing respiratory protection for workers for use during centrifugation or other procedures with increased potential for inadvertent sample release. Acceptable methods of respiratory protection include a properly fit tested NIOSH approved filter respirator (N-95 or higher); or powered air-purifying respirators (PAPRs) equipped with high efficiency particulate air (HEPA) filters. Accurate fit testing is a key component of effective respirator use. Personnel who cannot wear fitted respirators because of facial hair or other fit-limitations should wear loose fitting hooded or helmeted PAPRs.

[0134] Consideration may also be given to referral of specimens to a suitably equipped reference laboratory.

[0135] To further illustrate, prior to analyzing the target nucleic acids described herein, those nucleic acids may be purified or isolated from samples that typically include complex mixtures of different components. Cells in collected samples are typically lysed to release the cell contents. For example, cells suspected of being infected with SARS-CoV in the particular sample can be lysed by contacting them with various enzymes, chemicals, and/or lysed by other approaches known in the art. In some embodiments, nucleic acids are analyzed directly in the cell lysate. In other embodiments, nucleic acids are further purified or extracted from cell lysates prior to detection. Essentially any nucleic acid extraction methods can be used to purify nucleic acids in the samples utilized in the methods of the present invention. Exemplary

techniques that can be used to purifying nucleic acids include, e.g., affinity chromatography, hybridization to probes immobilized on solid supports, liquid-liquid extraction (e.g., phenol-chloroform extraction, etc.), precipitation (e.g., using ethanol, etc.), extraction with filter paper, extraction with micelle-forming reagents (e.g., cetyl-trimethyl-ammonium-bromide, etc.), binding to immobilized intercalating dyes (e.g., ethidium bromide, acridine, etc.), adsorption to silica gel or diatomic earths, adsorption to magnetic glass particles or organo silane particles under chaotropic conditions, and/or the like. Sample processing is also described in, e.g., US Pat. Nos. 5,155,018, 6,383,393, and 5,234,809, which are each incorporated by reference.

[0136] To further exemplify, unmodified nucleic acids can bind to a material with a silica surface. Many of these processes that are optionally adapted for use in the performing the methods of the present invention are described in the art. To illustrate, Vogelstein et al. (1979) Proc. Natl. Acad. Sci. USA 76:615-619, which is incorporated by reference, describes the purification of nucleic acids from agarose gels in the presence of sodium iodide using ground flint glass. Marko et al. (1982) Anal. Biochem. 121:382-387, which is incorporated by reference, describes the purification of nucleic acids from bacteria on glass dust in the presence of sodium perchlorate. In DE-A 3734442, which is incorporated by reference, nucleic acids are isolated on glass fiber filters. The nucleic acids bound to these glass fiber filters are washed and then eluted with a methanol-containing Tris/EDTA buffer. A similar procedure is described in Jakobi et al. (1988) Anal. Biochem. 175:196-201, which is incorporated by reference. In particular, Jakobi et al. describes the selective binding of nucleic acids to glass surfaces in chaotropic salt solutions and separating the nucleic acids from contaminants, such as agarose, proteins, and cell residue. To separate the glass particles from the contaminants, the particles can be centrifuged or fluids can be drawn through the glass fiber filters. In addition, the use of magnetic particles to immobilize nucleic acids after precipitation by adding salt and ethanol is described in, e.g., Alderton et al. (1992) Anal. Biochem. 201:166-169 and PCT/GB91/00212, which are both incorporated by reference. In this procedure, the nucleic acids are agglutinated along with the magnetic particles. The agglutinate is separated from the original solvent by applying a magnetic field and performing one or more washing steps. After at least one wash step, the nucleic acids are typically dissolved in a Tris buffer.

[0137] Magnetic particles in a porous glass matrix that is covered with a layer that includes, e.g., streptavidin can also be utilized in certain embodiments of the invention. These particles can be used, e.g., to isolate biotin-conjugated nucleic acids and proteins. Ferrimagnetic, ferromagnetic, and superparamagnetic particles are also optionally utilized. Magnetic glass particles and related methods that can be adapted for using in performing the methods described herein are also described in, e.g., WO 01/37291, which is incorporated by reference.

[0138] One of the most powerful and basic technologies for deriving and detecting nucleic acids is nucleic acid amplification. In the present invention, amplification of nucleic acids of interest typically precedes or is concurrent with the detection of that DNA. In addition, the oligonucleotides described herein are also optionally amplified, e.g., following chemical synthesis or the like. In some embodiments, detectable signals are amplified, e.g., using branched nucleic acid or other signal amplification formats known in the art.

[0139] Amplification methods that are optionally utilized or adapted for use with the oligonucleotides and methods described herein include, e.g., various polymerase or ligase mediated amplification methods, such as the polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR), the ligase chain reaction (LCR), strand displacement amplification (SDA), nucleic acid sequence-based amplification (NASBA), rolling circle amplification (RCA), and/or the like. Details regarding the use of these and other amplification methods can be found in various articles and/or any of a variety of standard texts, including, e.g., Berger, Sambrook, Ausubel, and PCR Protocols A Guide to Methods and Applications (Innis et al. eds) Academic Press, Inc., San Diego, CA (1990) (Innis), Schweitzer et al. (2001) "Combining nucleic acid amplification and detection," Curr Opin Biotechnol. 12(1):21-27, all of which are incorporated by reference. Many available biology texts also have extended discussions regarding PCR and related amplification methods. Nucleic acid amplification is also described in, e.g., Mullis et al., (1987) U.S. Patent No. 4,683,202 and Sooknanan and Malek (1995) Biotechnology 13:563, which are both incorporated by reference. Improved methods of amplifying large nucleic acids by PCR are summarized in Cheng et al. (1994) Nature 369:684, which is incorporated by reference.

In certain embodiments, duplex PCR is utilized to amplify target nucleic acids. Duplex PCR amplification is described further in, e.g., Gabriel et al. (2003) "Identification of human remains by immobilized sequence-specific oligonucleotide analysis of mtDNA hypervariable regions I and II," Croat. Med. J. 44(3)293 and La et al. (2003) "Development of a duplex PCR assay for detection of Brachyspira hyodysenteriae and Brachyspira pilosicoli in pig feces," J. Clin. Microbiol. 41(7):3372, which are both incorporated by reference. Optionally, labeled primers (e.g., biotinylated primers, Scorpion primers, etc.) are utilized to amplify nucleic acids in a sample, e.g., to facilitate the detection of amplicons and the like. Scorpion primers are also described in, e.g., Whitcombe et al. (1999) "Detection of PCR products using self-probing amplicons and fluorescence" Nat Biotechnol. 17(8):804-807, which is incorporated by reference. Labeling is described further herein.

[0140] Amplicons are optionally recovered and purified from other reaction components by any of a number of methods well known in the art, including electrophoresis, chromatography, precipitation, dialysis, filtration, and/or centrifugation. Aspects of nucleic acid purification are described in, e.g., Douglas et al., DNA Chromatography, Wiley, John & Sons, Inc. (2002), and Schott, Affinity Chromatography: Template Chromatography of Nucleic Acids and Proteins, Chromatographic Science Series, #27, Marcel Dekker (1984), all of which are incorporated by reference. In certain embodiments, amplicons are not purified prior to detection. The detection of amplicons is described further below.

NESTED PCR

[0141] The present invention provides methods for detecting SARS-CoV in samples and typing the coronavirus. An exemplary method is as follows:

(a) Nested PCR using two sets of amplification primers. The target DNA sequence of one set of primer (termed "inner" primers) is located within the target sequence of the second set of primers (termed "outer" primers). In practice, a standard PCR reaction is first run with the patient sample using the "outer primers". Then a second PCR reaction is run with the "inner primers" using the product of the first reaction as the amplification target. This procedure increases the sensitivity of the assay by reamplifying the product of the first reaction in a second reaction. The

specificity of the assay is increased because the inner primers amplify only if the first PCR reaction yielded a specific product. A schematic depiction of a nested PCR procedure is shown in Figure 3.

(b) Treating the sample with consensus SARS virus primer nucleic acids, an agent for polymerization, and deoxynucleoside 5'-triphosphates under hybridizing conditions, in which consensus SARS virus primers are mixtures of oligonucleotides that comprise at least one pair of primers sufficiently complementary to the target region of the SARS-CoV genome to hybridize therewith such that extension products or amplicons are synthesized from at least one member of the pair. When separated from complementary strands, the amplicons can serve as templates for synthesis of extension products or amplicons of the other member of the pair of primer nucleic acids;

(c) Treating the sample under denaturing conditions to separate the primer extension products or amplicons from their templates to provide single-stranded molecules;

(d) Treating the products of step (b) with the consensus primers of the step (a) under conditions such that additional primer extension products are synthesized using the single-stranded molecules produced in step (c) as a template;

(e) Repeating steps (b) - (d) to synthesize detectable amounts of target SARS-CoV amplicons to providing amplified SARS-CoV sequences;

(f) Determining if amplification has occurred by treating the reaction mixture prepared in step (d) under hybridizing conditions with a generic probe and determining if hybridization and amplification has occurred; and,

(g) Hybridizing a type-specific DNA probe to the amplified DNA and determining if hybridization has occurred.

[0142] Amplification of nucleic acids by the polymerase chain reaction is also described in U.S. Pat. Nos. 4,683,202 and 4,683,195, which are both incorporated by reference. In general, PCR amplification of nucleic acids involves repeated cycles of heat-denaturing the nucleic acids, annealing oligonucleotide primers to sequences that flank the nucleic acid segment to be amplified, and extending the annealed primers with a polymerase. The primers hybridize to opposite strands of the target sequence and are oriented such that nucleic acid synthesis by the polymerase proceeds across the region

between the primers, effectively doubling the amount of the target nucleic acid synthesized in the previous cycle. This results in the exponential accumulation of the target nucleic acids.

[0143] The choice of primers for use in PCR typically determines the specificity of the amplification reaction. In the amplification steps of the method of the present invention, "consensus" primers are used that will amplify SARS-CoV sequences present in a sample, regardless of type. The consensus primers of the invention can include degenerate primers, mixtures of the oligonucleotides synthesized so that any one several nucleotides can be incorporated into a primer are sufficiently complementary to all types of SARS-CoV to amplify a nucleic acid sequence of any SARS-CoV present in the sample.

[0144] The present invention may also lead to the discovery of previously unknown strains or types of SARS-CoVs.

5'-NUCLEASE REACTIONS

[0145] The oligonucleotides of the invention can also be utilized as 5'-nuclease probes for the real-time detection of SARS-CoV in 5'-nuclease PCR reactions.

[0146] 5'-nuclease reactions typically use 5'-nuclease probes to detect specific PCR products by employing the 5'-->3' exonuclease activity of, e.g., *Taq* DNA polymerase. In certain embodiments, 5'-nuclease probes are blocked to prohibit extension at their 3' ends. 5'-nuclease probes typically include site-specific sequences and are labeled with a fluorescent reporter dye and a fluorescent quencher dye. Typically, 5'-nuclease probes hybridize to target sequences downstream from the 3' ends of primer sequences used for the PCR reactions. During PCR the 5'-nuclease probes hybridizes to its complementary single strand nucleic sequence within the target region. When amplification occurs 5'-nuclease probes are degraded due to the 5'-->3' exonuclease activity of, e.g., *Taq* DNA polymerase, thereby separating the quencher from the reporter during extension. Due to the release of the quenching effect on the reporter, the detectable fluorescence intensity of the reporter dye increases. During the amplification process this light emission generally increases exponentially. Light emission from the reporter dye is typically monitored spectrophotometrically. Because

increase of the fluorescence intensity of the reporter dye is only achieved when probe hybridization and amplification of the target sequence has occurred, 5'-nuclease assays offer sensitive methods (viral nucleic acids at a concentration of only a few cfu/ml can be detected) to determine the presence or absence of specific sequences. Therefore, this technique is particularly useful in diagnostic applications, such as the screening of samples for the detection of SARS-CoV in clinical samples.

[0147] Exemplary probes useful for detection of the SARS-CoV in samples in 5'-nuclease reactions include, e.g., oligonucleotides having sequences corresponding to SEQ ID NOS: 17 and 21. To illustrate, a 5'-nuclease probe can incorporate a FAM fluorophore at the 5' end and the TAMRA quencher at the 3' end. To further illustrate, a 5'-nuclease probe with a sequence corresponding to SEQ ID NO: 21 is optionally used in real-time PCR reactions with primer nucleic acids having sequences corresponding to SEQ ID NO: 11 and SEQ ID NO: 20 in certain embodiments of the invention.

[0148] Example conditions optionally used in 5'-nuclease nuclease reactions to detect SARS-CoV in samples are shown below. Reaction Conditions II are generally more useful if the target nucleic acid is expected to be present in a relatively high proportion. Optimization of reaction conditions is considered within the skill of the ordinary practitioner of the art.

REACTION CONDITIONS I

2.5 µl 10X TaqMan® A Buffer	**1X**
3.0 µl 25mM $MgCl_2$	**3mM**
.75 µl 10mM dATP	**300 µM**
.75 µl 10mM dCTP	**300 µM**
.75 µl 10mM dGTP	**300 µM**

.75 µl 20mM dUTP	600 µM
.075 µl 100µM forward primer	300 nM
.225 µl 100µM reverse primer	900 nM
.25 µl 10µM probe (SEQ ID NO: 17 or 21)	100nM
.125 µl Amplitaq Gold® (5 U/µl)	.025 U/µl
.25 µl AmpErase® UNG (1 U/µl)	.01 U/µl
13.575 µl Nuclease-free water	
2.0 µl DNA standard/sample	
total volume = 25 µl	

CYCLING CONDITIONS

50°C for 2 min

60°C for 30 min

95°C for 5 min

40 cycles of 95°C 15 and 60°C for 1 min

25°C for 2 min

REACTION CONDITIONS II

5 µl 10X TaqMan® Buffer A	1X
11 µl 25mM $MgCl_2$	5.5mM

11 µl 25mM $MgCl_2$	5.5mM
1 µl 10mM dATP	200 µM
1 µl 10mM dCTP	200 µM
1 µl 10mM dGTP	200 µM
1 µl 20mM dUTP	400 µM
1 µl 10µM forward primer	200 nM
1 µl 10µM reverse primer	200 nM
1 µl 10µM probe (SEQ ID NO: 17 or 21)	100nM
.5 AmpliTaq Gold® (5 U/µl)	.05 U/µl
.5 µl AmpErase® UNG (1 U/µl)	.01 U/µl
1.25 µl 2% glycerin	0.05%
23.08 µl RNase-free water	
1.67 µl DNA standard/sample	
total volume = 50 µl	

CYCLING CONDITIONS

50°C for 2 min

95°C for 5 min

40 cycles of 95°C for 15 sec and 60°C for 1 min

25°C for 2 min

[0149] An exhaustive manual for conducting RT-PCR using 5'-nuclease reactions is available from Applied Biosystems, "TaqMan® One-Step RT-PCR Master Mix Reagents Kit Protocol", printed 4/2002, as part no. 4310299 Rev.C., hereby incorporated in its entirety by reference. The publication was available on the world wide web at ucl.ac.uk/wibr/2/services/reldocs/1steppcr.pdf as of April 20, 2004.

VII. OLIGONUCLEOTIDE ARRAYS

[0150] In certain embodiments of the invention, the oligonucleotides described herein are covalently or noncovalently attached to solid supports which are then contacted with samples comprising amplified and labeled nucleic acid from a subject. In other embodiments, the oligonucleotides of the invention are provided free in solution. Essentially any substrate material is optionally adapted for use in these aspects of the invention. In certain embodiments, for example, substrates are fabricated from silicon, glass, or polymeric materials (e.g., glass or polymeric microscope slides, silicon wafers, etc.). Suitable glass or polymeric substrates, including microscope slides, are available from various commercial suppliers, such as Fisher Scientific (Pittsburgh, PA) or the like. In some embodiments, solid supports utilized in the invention are membranes. Suitable membrane materials are optionally selected from, e.g. polyaramide membranes, polycarbonate membranes, porous plastic matrix membranes (e.g., POREX® Porous Plastic, etc.), porous metal matrix membranes, polyethylene membranes, poly(vinylidene difluoride) membranes, polyamide membranes, nylon membranes, ceramic membranes, polyester membranes, polytetrafluoroethylene (TEFLON®) membranes, woven mesh membranes, microfiltration membranes, nanofiltration membranes, ultrafiltration membranes, dialysis membranes, composite membranes, hydrophilic membranes, hydrophobic

membranes, polymer-based membranes, a non-polymer-based membranes, powdered activated carbon membranes, polypropylene membranes, glass fiber membranes, glass membranes, nitrocellulose membranes, cellulose membranes, cellulose nitrate membranes, cellulose acetate membranes, polysulfone membranes, polyethersulfone membranes, polyolefin membranes, or the like. Many of these membranous materials are widely available from various commercial suppliers, such as, P.J. Cobert Associates, Inc. (St. Louis, MO), Millipore Corporation (Bedford, MA), or the like. Other exemplary solid supports that are optionally utilized include, e.g., ceramics, metals, resins, gels, plates, beads, microbeads (e.g., magnetic microbeads, etc.), tubes (e.g., microtubes, etc.), whiskers, fibers, combs, single crystals, and self-assembling monolayers.

[0151] The oligonucleotides of the invention are directly or indirectly (e.g., via linkers, such as bovine serum albumin (BSA) or the like) attached to the supports, e.g., by any available chemical or physical method. A wide variety of linking chemistries are available for linking molecules to a wide variety of solid supports. More specifically, nucleic acids may be attached to the solid support by covalent binding such as by conjugation with a coupling agent or by non-covalent binding such as electrostatic interactions, hydrogen bonds or antibody-antigen coupling, or by combinations thereof. Typical coupling agents include biotin/avidin, biotin/streptavidin, *Staphylococcus aureus* protein A/IgG antibody F_c fragment, and streptavidin/protein A chimeras (Sano et al. (1991) Bio/Technology 9:1378, which is incorporated by reference), or derivatives or combinations of these agents. Nucleic acids may be attached to the solid support by a photocleavable bond, an electrostatic bond, a disulfide bond, a peptide bond, a diester bond or a combination of these bonds. Nucleic acids are also optionally attached to solid supports by a selectively releasable bond such as 4,4'-dimethoxytrityl or its derivative. Derivatives which have been found to be useful include 3 or 4 [bis-(4-methoxyphenyl)]-methyl-benzoic acid, N-succinimidyl-3 or 4 [bis-(4-methoxyphenyl)]-methyl-benzoic acid, N-succinimidyl-3 or 4 [bis-(4-methoxyphenyl)]-hydroxymethyl-benzoic acid, N-succinimidyl-3 or 4 [bis-(4-methoxyphenyl)]-chloromethyl-benzoic acid, and salts of these acids.

[0152] As referred to above, oligonucleotides are optionally attached to solid supports via linkers between the nucleic acids and the solid support. Useful linkers include a coupling agent, as described above for binding to other or additional coupling partners, or to render the attachment to the solid support cleavable.

[0153] Cleavable attachments can be created by attaching cleavable chemical moieties between the oligonucleotides and the solid support including, e.g., an oligopeptide, oligonucleotide, oligopolyamide, oligoacrylamide, oligoethylene glycerol, alkyl chains of between about 6 to 20 carbon atoms, and combinations thereof. These moieties may be cleaved with, e.g., added chemical agents, electromagnetic radiation, or enzymes. Exemplary attachments cleavable by enzymes include peptide bonds which can be cleaved by proteases, and phosphodiester bonds which can be cleaved by nucleases.

[0154] Chemical agents such as β-mercaptoethanol, dithiothreitol (DTT) and other reducing agents cleave disulfide bonds. Other agents which may be useful include oxidizing agents, hydrating agents and other selectively active compounds. Electromagnetic radiation such as ultraviolet, infrared and visible light cleave photocleavable bonds. Attachments may also be reversible, e.g., using heat or enzymatic treatment, or reversible chemical or magnetic attachments. Release and reattachment can be performed using, e.g., magnetic or electrical fields.

[0155] Array based hybridization is particularly suitable for detecting SARS-CoV nucleic acids, as it can be used to detect the presence of many amplicons simultaneously. A number of array systems have been described and can be adapted for use with the present invention, including those available from commercial suppliers such as Affymetrix, Inc. (Santa Clara, CA, USA) and the like. Aspects of array construction and use are also described in, e.g., Sapolsky et al. (1999) "High-throughput polymorphism screening and genotyping with high-density oligonucleotide arrays." Genetic Analysis: Biomolecular Engineering 14:187-192; Lockhart (1998) "Mutant yeast on drugs" Nature Medicine 4:1235-1236; Fodor (1997) "Genes, Chips and the Human Genome." FASEB Journal 11:A879; Fodor (1997) "Massively Parallel Genomics" Science 277: 393-395; and Chee et al. (1996) "Accessing Genetic

Information with High-Density DNA Arrays" Science 274:610-614, all of which are incorporated by reference.

VIII. NUCLEIC ACID HYBRIDIZATION

[0156] Hybridization of oligonucleotides to their target SARS-CoV nucleic acids can be accomplished by choosing the appropriate hybridization conditions. The stability of the probe:target nucleic acid hybrid is typically selected to be compatible with the assay and washing conditions so that stable, detectable hybrids form only between the probes and target SARS-CoV nucleic acids. Manipulation of one or more of the different assay parameters determines the exact sensitivity and specificity of a particular hybridization assay.

[0157] More specifically, hybridization between complementary bases of DNA, RNA, PNA, or combinations of DNA, RNA and PNA, occurs under a wide variety of conditions that vary in temperature, salt concentration, electrostatic strength, buffer composition, and the like. Examples of these conditions and methods for applying them are described in, e.g., Tijssen (1993), *supra*, and Hames and Higgins, *supra*. Hybridization generally takes place between about 0°C and about 70°C, for periods of from about one minute to about one hour, depending on the nature of the sequence to be hybridized and its length. However, it is recognized that hybridizations can occur in seconds or hours, depending on the conditions of the reaction. To illustrate, typical hybridization conditions for a mixture of two 20-mers is to bring the mixture to 68°C, followed by cooling to room temperature (22°C) for five minutes or at very low temperatures such as 2°C in 2 microliters. Hybridization between nucleic acids may be facilitated using buffers such as Tris-EDTA (TE), Tris-HCl and HEPES, salt solutions (e.g. NaCl, KCl, $CaCl_2$), or other aqueous solutions, reagents and chemicals. Examples of these reagents include single-stranded binding proteins such as Rec A protein, T4 gene 32 protein, *E. coli* single-stranded binding protein and major or minor nucleic acid groove binding proteins. Other examples of such reagents and chemicals include divalent ions, polyvalent ions and intercalating substances such as ethidium bromide, actinomycin D, psoralen, and angelicin.

IX. DETECTION AND OLIGONUCLEOTIDE VARIATIONS

[0158] As referred to above, amplified target SARS-CoV nucleic acid in the samples utilized in the methods of the invention is optionally labeled to permit detection of oligonucleotide-target hybridization duplexes. In general, a label can be any moiety that can be attached, e.g., to a primer utilized for amplification and provide a detectable signal (e.g., a quantifiable signal). Labels may be attached to a primer directly or indirectly by a variety of techniques known in the art. Depending on the type of label used, the label can be attached to a terminal (5' or 3' end of the primer) or a non-terminal nucleotide, and can be attached indirectly through linkers or spacer arms of various sizes and compositions. Using commercially available phosphoramidite reagents, one can produce oligomers containing functional groups (e.g., thiols or primary amines) at either the 5' or 3' terminus via an appropriately protected phosphoramidite, and can label such oligonucleotides using protocols described in, for example, PCR Protocols: A Guide to Methods and Applications (Innis et al, eds. Academic Press, Inc. (1990)). In one embodiment, the label consists of a biotin molecule covalently bound to the primer at the 5' end. The term "biotinylated primer" refers to a primer with one or more biotin molecules bound either directly to the primer or indirectly through intervening linker molecules.

[0159] To further illustrate, detection of oligonucleotide-target hybridization duplexes is optionally by a chemiluminescent assay using a luminol-based reagent as described in, e.g., Whitehead, et al. (1983) Nature 30(5):158, which is incorporated by reference, and available commercially. Following hybridization of the oligonucleotide with the labeled target DNA, the biotin molecule attached to the target DNA is conjugated, e.g., to streptavidin-horseradish peroxidase (SA-HRP). Alternatively, the target DNA can be labeled with horseradish peroxidase directly, thereby eliminating the separate conjugation step. In either case, subsequent oxidation of luminol by the horseradish peroxidase enzyme results in the emission of photons, which is then detected, e.g., on standard autoradiography film. The intensity of the signal is a function of DNA quantity. A series of DNA standards containing known amounts of DNA are typically assayed along with one or more unknown samples. The signal intensities of the known DNA standards allow an empirical determination of the functional relationship between signal intensity and DNA quantity, which enables the

quantitation of the unknown samples. Many other methods of detection are also optionally utilized to perform the methods of the invention and are referred to in the references cited herein and/or generally known in the art.

[0160] Any available method for detecting SARS-CoV amplicons can be used in the present invention. Common approaches include real time amplification detection with molecular beacons or 5'-nuclease probes, detection of intercalating dyes, detection of labels incorporated into the amplification probes or the amplified nucleic acids themselves, e.g., following electrophoretic separation of the amplification products from unincorporated label), hybridization based assays (e.g., array based assays) and/or detection of secondary reagents that bind to the nucleic acids.

[0161] To further illustrate, a molecular beacon or a 5'-nuclease probe is optionally designed to include a oligonucleotide of the invention (i.e., is selected from SEQ ID NOS: 1-12 and 15-24) or complements thereto), which molecular beacon or 5'-nuclease probe can be used to detect SARS-CoV amplicons. Molecular beacons or 5'-nuclease probes are described further below. Details on these general approaches are found in the references cited herein, e.g., Sambrook and Ausubel. Additional labeling strategies for labeling nucleic acids and corresponding detection strategies can be found, e.g., in Haugland (2003) Handbook of Fluorescent Probes and Research Chemicals Ninth Edition by Molecular Probes, Inc. (Eugene, OR), which is incorporated by reference.

[0162] Molecular beacons (MBs) are oligonucleotides designed for real time detection and quantification of target nucleic acids (e.g., target SARS-CoV amplicons). The 5' and 3' termini of MBs collectively comprise a pair of moieties which confers the detectable properties of the MB. One of the termini is attached to a fluorophore and the other is attached to a quencher molecule capable of quenching a fluorescent emission of the fluorophore. For example, one example fluorophore-quencher pair can use a fluorophore such as EDANS or fluorescein, e.g., on the 5'-end and a quencher such as Dabcyl, e.g., on the 3'-end. When the MB is present free in solution, i.e., not hybridized to a second nucleic acid, the stem of the MB is stabilized by complementary base pairing. This self-complementary pairing results in a "hairpin loop" structure for the MB in which the fluorophore and the quenching moieties are proximal to one

another. In this confirmation, the fluorescent moiety is quenched by the fluorophore. The loop of the molecular beacon typically comprises an oligonucleotide described herein (i.e., is selected from SEQ ID NOS: 1-12 and 15-24 or complements thereto) and is accordingly complementary to a sequence to be detected in the target SARS-CoV nucleic acid, such that hybridization of the loop to its complementary sequence in the target forces disassociation of the stem, thereby distancing the fluorophore and quencher from each other. This results in unquenching of the fluorophore, causing an increase in fluorescence of the MB.

[0163] Details regarding standard methods of making and using MBs are well established in the literature and MBs are available from a number of commercial reagent sources. Further details regarding methods of MB manufacture and use are found, e.g., in Leone et al. (1995) "Molecular beacon probes combined with amplification by NASBA enable homogenous real-time detection of RNA," Nucleic Acids Res. 26:2150-2155; Hsuih et al. (1997) "Novel, ligation-dependent PCR assay for detection of hepatitis C in serum" J Clin Microbiol 34:501-507; Kostrikis et al. (1998) "Molecular beacons: spectral genotyping of human alleles" Science 279:1228-1229; Sokol et al. (1998) "Real time detection of DNA:RNA hybridization in living cells" Proc. Natl. Acad. Sci. U.S.A. 95:11538-11543; Tyagi et al. (1998) "Multicolor molecular beacons for allele discrimination" Nature Biotechnology 16:49-53; Fang et al. (1999) "Designing a novel molecular beacon for surface-immobilized DNA hybridization studies" J. Am. Chem. Soc. 121:2921-2922; and Marras et al. (1999) "Multiplex detection of single-nucleotide variation using molecular beacons" Genet. Anal. Biomol. Eng. 14:151-156, all of which are incorporated by reference. Aspects of MB construction and use are also found in patent literature, such as U.S. Pat. No. 5,925,517 (July 20, 1999) to Tyagi et al. entitled "Detectably labeled dual conformation oligonucleotide probes, assays and kits;" U.S. Pat. No. 6,150,097 to Tyagi et al (November 21, 2000) entitled "Nucleic acid detection probes having non-FRET fluorescence quenching and kits and assays including such probes" and U.S. Pat. No. 6,037,130 to Tyagi et al (March 14, 2000), entitled "Wavelength-shifting probes and primers and their use in assays and kits," all of which are incorporated by reference.

[0164] MB components (e.g., oligos, including those labeled with fluorophores or quenchers) can be synthesized using conventional methods. Some of these methods are described further above. For example, oligonucleotides or peptide nucleic acids (PNAs) can be synthesized on commercially available automated oligonucleotide/PNA synthesis machines using standard methods. Labels can be attached to the oligonucleotides or PNAs either during automated synthesis or by post-synthetic reactions which have been described before *see, e.g.,* Tyagi and Kramer (1996), supra. Aspects relating to the synthesis of functionalized oligonucleotides can also be found in Nelson, et al. (1989) "Bifunctional Oligonucleotide Probes Synthesized Using A Novel CPG Support Are Able To Detect Single Base Pair Mutations" Nucleic Acids Res. 17:7187-7194, which is incorporated by reference. Labels/ quenchers can be introduced to the oligonucleotides or PNAs, e.g., by using a controlled-pore glass column to introduce, e.g., the quencher (e.g., a 4-dimethylaminoazobenzene-4'-sulfonyl moiety (DABSYL). For example, the quencher can be added at the 3' end of oligonucleotides during automated synthesis; a succinimidyl ester of 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL) can be used when the site of attachment is a primary amino group; and 4-dimethylaminophenylazophenyl-4'-maleimide (DABMI) can be used when the site of attachment is a sulphydryl group. Similarly, fluorescein can be introduced in the oligonucleotides, either using a fluorescein phosphoramadite that replaces a nucleoside with fluorescein, or by using a fluorescein dT phosphoramadite that introduces a fluorescein moiety at a thymidine ring via a linker. To link a fluorescein moiety to a terminal location, iodoacetoamidofluorescein can be coupled to a sulphydryl group. Tetrachlorofluorescein (TET) can be introduced during automated synthesis using a 5'-tetrachloro-fluorescein phosphoramadite. Other reactive fluorophore derivatives and their respective sites of attachment include the succinimidyl ester of 5-carboxyrhodamine-6G (RHD) coupled to an amino group; an iodoacetamide of tetramethylrhodamine coupled to a sulphydryl group; an isothiocyanate of tetramethylrhodamine coupled to an amino group; or a sulfonylchloride of Texas red coupled to a sulphydryl group. During the synthesis of these labeled components, conjugated oligonucleotides or PNAs can be purified, if desired, e.g., by high pressure liquid chromatography or other methods.

[0165] A variety of commercial suppliers produce standard and custom molecular beacons, including Cruachem (cruachem.com), Oswel Research Products Ltd. (UK; oswel.com), Research Genetics (a division of Invitrogen, Huntsville AL (resgen.com)), the Midland Certified Reagent Company (Midland, TX mcrc.com) and Gorilla Genomics, LLC (Alameda, CA). A variety of kits, which utilize molecular beacons are also commercially available, such as the Sentinel™ Molecular Beacon Allelic Discrimination Kits from Stratagene (La Jolla, CA) and various kits from Eurogentec SA (Belgium, eurogentec.com) and Isogen Bioscience BV (The Netherlands, isogen.com).

[0166] In certain embodiments, as also referred to above, a real time PCR assay system that includes one or more 5'-nuclease probes is used for detecting amplified SARS-CoV nucleic acids. These systems operate by using the endogenous nuclease activity of certain polymerases to cleave a quencher or label free from an oligonucleotide of the invention that comprises the quencher and label, resulting in unquenching of the label. The polymerase only cleaves the quencher or label upon initiation of replication, i.e., when the oligonucleotide is bound to the template and the polymerase extends the primer. Thus, an appropriately labeled oligonucleotide and polymerase comprising the appropriate nuclease activity can be used to detect a SARS-CoV nucleic acid of interest. Real time PCR product analysis by, e.g., FRET or the like (and related real time reverse-transcription PCR) provides a well-known technique for real time PCR monitoring that has been used in a variety of contexts, which can be adapted for use with the probes and methods described herein (*see*, Laurendeau et al. (1999) "TaqMan PCR-based gene dosage assay for predictive testing in individuals from a cancer family with INK4 locus haploinsufficiency" Clin Chem 45(7):982-6; Laurendeau et al. (1999) "Quantitation of MYC gene expression in sporadic breast tumors with a real-time reverse transcription-PCR assay" Clin Chem 59(12):2759-65; and Kreuzer et al. (1999) "LightCycler technology for the quantitation of bcr/abl fusion transcripts" Cancer Research 59(13):3171-4, all of which are incorporated by reference). To further illustrate, exemplary 5'-nuclease probes of the invention include 5'-(6-FAM)-AGCTAACGAGTGTGCGCAAGTATTAAGTGAGATG-(TAMRA)(Phosphate)-3' (SEQ ID NO: 27) and 5'-(6-FAM)-AGAGCCATGCCTAACAT(NFQ)-3' (SEQ ID NO: 28).

X. SYSTEMS

[0167] The invention also provides a system for detecting SARS-CoV in a sample. The system includes one or more nucleic acid detection reagents as described herein (e.g., probe nucleic acids, sequence specific antibodies, etc.). In certain embodiments, the nucleic acid detection reagents are arrayed on a solid support, whereas in others, they are provided in one or more containers, e.g., for assays performed in solution. The system also includes at least one detector (e.g., a spectrometer, etc.) that detects binding between nucleic acids and/or amplicons thereof from the sample and the nucleic acid detection reagent. Other detectors are described further below. In addition, the system also includes at least one controller operably connected to the detector. The controller includes one or more instructions sets that correlate the binding detected by the detector with a presence of SARS-CoV in the sample.

[0168] In some embodiments, at least one container includes the nucleic acid detection reagent. In these embodiments, the system optionally further includes at least one thermal modulator operably connected to the container to modulate temperature in the container, and/or at least one fluid transfer component (e.g., an automated pipettor, etc.) that transfers fluid to and/or from the container, e.g., for performing one or more nucleic acid amplification techniques in the container, etc.

[0169] Exemplary commercially available systems that are optionally utilized to detect SARS-CoV nucleic acids using the nucleic acid detection reagents described herein (e.g., oligonucleotides comprising sequences selected from the group consisting of: SEQ ID NOS: 1-12 and 15-24 or complements thereto, sequence specific antibodies, etc.) include, e.g., a LightCycler™ system (e.g., LightCycler™ RNA Master SYBR Green Detection) or a COBAS AMPLICOR® Analyzer, which are available from Roche Diagnostics Corporation (Indianapolis, IN), a LUMINEX 100™ system, which is available from the Luminex Corporation (Austin, TX), an ABI PRISM® Sequence Detection System, which is available from Applied Biosystems (Foster City, CA), and the like.

[0170] The invention further provides a computer or computer readable medium that includes a data set that comprises a plurality of character strings that

correspond to a plurality of sequences that correspond to one or more of: SEQ ID NOS: 1-12 and 15-24, a substantially identical variant thereof wherein the variant has at least 90% sequence identity to one of SEQ ID NOS: 1-12 and 15-24, and complements of SEQ ID NOS: 1-12 and 15-24 and the variant. Typically, the computer or computer readable medium further includes an automatic synthesizer coupled to an output of the computer or computer readable medium. The automatic synthesizer accepts instructions from the computer or computer readable medium, which instructions direct synthesis of, e.g., one or more probe nucleic acids that correspond to one or more character strings in the data set. Exemplary systems and system components are described further below.

[0171] Detectors are structured to detect detectable signals produced, e.g., in or proximal to another component of the system (e.g., in container, on a solid support, etc.). Suitable signal detectors that are optionally utilized, or adapted for use, in these systems detect, e.g., fluorescence, phosphorescence, radioactivity, absorbance, refractive index, luminescence, or the like. Detectors optionally monitor one or a plurality of signals from upstream and/or downstream of the performance of, e.g., a given assay step. For example, the detector optionally monitors a plurality of optical signals, which correspond in position to "real time" results. Example detectors or sensors include photomultiplier tubes, CCD arrays, optical sensors, temperature sensors, pressure sensors, pH sensors, conductivity sensors, scanning detectors, or the like. Each of these as well as other types of sensors is optionally readily incorporated into the systems described herein. Optionally, the systems of the present invention include multiple detectors.

[0172] More specific exemplary detectors that are optionally utilized in these systems include, e.g., a resonance light scattering detector, an emission spectroscope, a fluorescence spectroscope, a phosphorescence spectroscope, a luminescence spectroscope, a spectrophotometer, a photometer, and the like. Various synthetic components are also utilized, or adapted for, use in the systems of the invention including, e.g., automated nucleic acid synthesizers, e.g., for synthesizing the oligonucleotides described herein. Detectors and synthetic components that are optionally included in the systems of the invention are described further in, e.g., Skoog

et al., Principles of Instrumental Analysis, 5th Ed., Harcourt Brace College Publishers (1998) and Currell, Analytical Instrumentation: Performance Characteristics and Quality, John Wiley & Sons, Inc. (2000), both of which are incorporated by reference.

[0173] The systems of the invention also typically include controllers that are operably connected to one or more components (e.g., detectors, synthetic components, thermal modulator, fluid transfer components, etc.) of the system to control operation of the components. More specifically, controllers are generally included either as separate or integral system components that are utilized, e.g., to receive data from detectors, to effect and/or regulate temperature in the containers, to effect and/or regulate fluid flow to or from selected containers, or the like. Controllers and/or other system components is/are optionally coupled to an appropriately programmed processor, computer, digital device, or other information appliance (e.g., including an analog to digital or digital to analog converter as needed), which functions to instruct the operation of these instruments in accordance with preprogrammed or user input instructions, receive data and information from these instruments, and interpret, manipulate and report this information to the user. Suitable controllers are generally known in the art and are available from various commercial sources.

[0174] Any controller or computer optionally includes a monitor, which is often a cathode ray tube ("CRT") display, a flat panel display (e.g., active matrix liquid crystal display, liquid crystal display, etc.), or others. Computer circuitry is often placed in a box, which includes numerous integrated circuit chips, such as a microprocessor, memory, interface circuits, and others. The box also optionally includes a hard disk drive, a floppy disk drive, a high capacity removable drive such as a writeable CD-ROM, and other common peripheral elements. Inputting devices such as a keyboard or mouse optionally provide for input from a user. These components are illustrated further below.

[0175] The computer typically includes appropriate software for receiving user instructions, either in the form of user input into a set of parameter fields, e.g., in a GUI, or in the form of preprogrammed instructions, e.g., preprogrammed for a variety of different specific operations. The software then converts these instructions to appropriate language for instructing the operation of one or more controllers to carry

out the desired operation. The computer then receives the data from, e.g., sensors/detectors included within the system, and interprets the data, either provides it in a user understood format, or uses that data to initiate further controller instructions, in accordance with the programming, e.g., such as controlling fluid flow regulators in response to fluid weight data received from weight scales or the like.

[0176] The computer can be, e.g., a PC (Intel x86 or Pentium chip-compatible DOS™, OS2™, WINDOWS™, WINDOWS NT™, WINDOWS95™, WINDOWS98™, WINDOWS2000™, WINDOWS XP™, LINUX-based machine, a MACINTOSH™, Power PC, or a UNIX-based (e.g., SUN™ work station) machine) or other common commercially available computer, which is known to one of skill in the art. Standard desktop applications such as word processing software (e.g., Microsoft Word™ or Corel WordPerfect™) and database software (e.g., spreadsheet software such as Microsoft Excel™, Corel Quattro Pro™, or database programs such as Microsoft Access™ or Paradox™) can be adapted to the present invention. Software for performing, e.g., controlling temperature modulators and fluid flow regulators is optionally constructed by one of skill using a standard programming language such as Visual basic, Fortran, Basic, Java, or the like.

[0177] Figures 4 and 5 are schematics showing representative example systems that include logic devices in which various aspects of the present invention may be embodied. As will be understood by practitioners in the art from the teachings provided herein, the invention is optionally implemented in hardware and/or software. In some embodiments, different aspects of the invention are implemented in either client-side logic or server-side logic. As will be understood in the art, the invention or components thereof may be embodied in a media program component (e.g., a fixed media component) containing logic instructions and/or data that, when loaded into an appropriately configured computing device, cause that device to perform according to the invention. As will also be understood in the art, a fixed media containing logic instructions may be delivered to a viewer on a fixed media for physically loading into a viewer's computer or a fixed media containing logic instructions may reside on a remote server that a viewer accesses through a communication medium in order to download a program component.

[0178] In particular, Figure 4 schematically illustrate computer **400** to which detector **402** and fluid transfer component **404** are operably connected. Optionally, detector **402** and/or fluid transfer component **404** is operably connected to computer **400** via a server (not shown in Figure 4). During operation, fluid transfer component **404** typically transfers fluids, such as sample aliquots comprising labeled SARS-CoV amplicons to nucleic acid detection reagent array **406**, e.g., comprising oligonucleotides, sequence specific antibodies, etc., as described herein, arrayed thereon. Thereafter, detector **402** typically detects detectable signals (e.g., fluorescent emissions, etc.) produced by labeled amplicons that hybridize with oligonucleotides attached to nucleic acid detection reagent array **406** after one or more washing steps are performed to wash away non-hybridized nucleic acids from nucleic acid detection reagent array **406** using fluid transfer component **404**. As additionally shown, thermal modulator **408** is also operably connected to computer **400**. Prior to performing a hybridization assay, target SARS-CoV nucleic acids can be amplified using labeled primer nucleic acids (e.g., primers comprising sequences selected from SEQ ID NOS: 1-12 and 15-24 or complements thereof). The amplicons of these amplification reactions are then typically transferred to nucleic acid detection reagent array **406** using fluid transfer component **404**, as described above, to perform the binding assay. In some embodiments, binding assays are performed concurrently with SARS-CoV nucleic acid amplification in thermal modulator **408** using, e.g., molecular beacons, 5'-nuclease probes, or the like that comprise sequences selected from the group consisting of: SEQ ID NOS: 1-12 and 15-24, a substantially identical variant thereof wherein the variant has at least 90% sequence identity to one of SEQ ID NOS: 1-12 and 15-24, and complements of SEQ ID NOS: 1-12 and 15-24 and the variant. In these embodiments, detector **402** detects detectable signals produced as the amplification reactions are performed using thermal modulator **408**.

[0179] Figure 5 schematically shows information appliance or digital device **500** that may be understood as a logical apparatus that can read instructions from media **502** and/or network port **504**, which can optionally be connected to server **506** having fixed media **508**. Digital device **500** can thereafter use those instructions to direct server or client logic, as understood in the art, to embody aspects of the invention. One type of logical apparatus that may embody the invention is a computer system as

illustrated in 500, containing CPU 510, optional input devices 512 and 514, disk drives 516 and optional monitor 518. Fixed media 502, or fixed media 508 over port 504, may be used to program such a system and may represent a disk-type optical or magnetic media, magnetic tape, solid state dynamic or static memory, or the like. In specific embodiments, the invention may be embodied in whole or in part as software recorded on this fixed media. Communication port 504 may also be used to initially receive instructions that are used to program such a system and may represent any type of communication connection. Optionally, the invention is embodied in whole or in part within the circuitry of an application specific integrated circuit (ACIS) or a programmable logic device (PLD). In such a case, the invention may be embodied in a computer understandable descriptor language, which may be used to create an ASIC, or PLD.

[0180] Figure 5 also includes automatic synthesizer 350, which is operably connected to digital device 500 via server 506. Optionally, automatic synthesizer 520 is directly connected to digital device 500. During operation, automatic synthesizer 520 typically receives instructions to synthesize one or more primers or probes that comprise a sequence selected from the group consisting of: SEQ ID NOS: 1-12 and 15-24, a substantially identical variant thereof wherein the variant has at least 90% sequence identity to one of SEQ ID NOS: 1-12 and 15-24, and complements of SEQ ID NOS: 1-12 and 15-24 and the variant, which are included in a data set comprised by, e.g., digital device 500 and/or a computer readable medium, such as fixed media 502 and/or 508.

XI. KITS

[0181] The nucleic acid detection reagents (e.g., primer nucleic acids, probe nucleic acids, etc.) employed in the methods of the present invention are optionally packaged into kits. As described herein, the nucleic acid detection reagents of the invention detectably bind to target SARS-CoV nucleic acids. In addition, the kits may also include suitably packaged reagents and materials needed for nucleic acid immobilization, hybridization, and/or detection, such solid supports, buffers, enzymes, and nucleic acid standards, as well as instructions for conducting the assay. Optionally, the nucleic acid detection reagents (e.g., oligonucleotides, sequence specific antibodies,

etc.) of the invention are provided already attached or otherwise immobilized on solid supports. As another option, nucleic acid detection reagents are provided free in solution in containers, e.g., for performing the detection methods of the invention in the solution phase. In some of these embodiments, nucleic acid detection reagents of the kits comprise labels and/or quencher moieties, such as when molecular beacons, 5'-nuclease probes, or the like comprise sequences selected from the group consisting of: SEQ ID NOS: 1-12 and 15-24, a substantially identical variant thereof wherein the variant has at least 90% sequence identity to one of SEQ ID NOS: 1-12 and 15-24, and complements of SEQ ID NOS: 1-12 and 15-24 and the variant. In certain embodiments, kits further include labeled primers for amplifying target SARS-CoV sequences in a sample.

[0182] The kit also includes one or more of: a set of instructions for contacting the nucleic acid detection reagents with nucleic acids from a sample or amplicons thereof and detecting binding between the nucleic acid detection reagents and SARS-CoV nucleic acids, or at least one container for packaging the nucleic acid detection reagents and the set of instructions. Exemplary solid supports include in the kits of the invention are optionally selected from, e.g., a plate, a microwell plate, a bead, a microbead, a tube (e.g., a microtube, etc.), a fiber, a whisker, a comb, a hybridization chip, a membrane, a single crystal, a ceramic layer, a self-assembling monolayer, or the like.

[0183] In some embodiments, the kit further includes at least one primer nucleic acid that is at least partially complementary to at least one segment of a SARS-CoV nucleic acid, e.g., for amplifying a segment of the SARS-CoV nucleic acid. In these embodiments, the kit typically further includes a set of instructions for amplifying one or more subsequences of that nucleic acid with the primer nucleic acids, at least one nucleotide incorporating biocatalyst (e.g., a polymerase), and one or more deoxyribonucleotides. In certain embodiments, the primer nucleic acids comprise at least one label (e.g., a fluorescent dye, a radioisotope, etc.). Suitable labels are described further herein. For example, the primer nucleic acid is optionally conjugated with biotin or a biotin derivative. In these embodiments, the kit typically further includes an enzyme conjugated with avidin or an avidin derivative, or streptavidin or a

streptavidin derivative, e.g., for effecting the detection of binding between the nucleic acid detection reagents of the invention and target nucleic acids. In these embodiments, the kit generally further includes at least one nucleotide incorporating biocatalyst (e.g., a polymerase, a ligase, or the like). In these embodiments, the kit typically also further comprising one or more nucleotides, e.g., for use in amplifying the target nucleic acids. Optionally, at least one of the nucleotides comprises a label. In some of these embodiments, the kits further include at least one pyrophosphatase (e.g., a thermostable pyrophosphatase), e.g., for use in minimizing pyrophosphorolysis, uracil N-glycosylase (UNG) (e.g., a thermostable UNG), e.g., for use in applications where protection against carry-over contamination is desirable.

XII. EXAMPLES

[0184] It is understood that the examples and embodiments described herein are for illustrative purposes only and are not intended to limit the scope of the claimed invention. It is also understood that various modifications or changes in light the examples and embodiments described herein will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

EXAMPLE 1: NESTED PCR DETECTION OF SARS CORONAVIRUS

[0185] The SARS Coronavirus Detection RT-PCT Diagnostic is specifically designed for detecting patients suspected of having SARS. Reagents are used for end-point PCR and the products analyzed by agarose gel electrophoresis. Reagents are designed to allow flexibility in the choice of reverse transcriptase and Taq polymerases.

[0186] Reagents provided (10 items):

1. Positive control (2.5 kb DNA fragment comprising the primer sequences) Neat
2. Positive control 1/10 dilution
3. Positive control 1/100 dilution
4. Negative control A (water or buffer solution alone)
5. Negative control B (cloning vector of the positive control fragment)
6. GISSARSOUT_F (10 μM) (SEQ ID NO: 11)

7. GISSARSOUT_R (10 μM)* (SEQ ID NO: 12 or 20)

8. GISSARSIN_F (10 μM) (SEQ ID NO: 13 (= SAR1S; see, Drosten et al. (2003) "Identification of a novel coronavirus in patients with severe acute respiratory syndrome," N. Engl. J. Med. 348:1967–1976), SEQ ID NO: 15 or 18)

9. GISSARSIN_R (10 μM) (SEQ ID NO: 14 (= SAR1As, see, Drosten et al. (2003), *supra*), SEQ ID NO: 16 or 19)

10. Sterile water for PCR

* GISSARSOUT_R is used both as the 3'-primer for first-strand cDNA synthesis and the first round PCR.

[0187] Reagents required but not provided:

1. *RNA extraction*

Recommended suppliers: Rneasy® kit (Qiagen)
or
TRIZOL® (Invitrogen)

2. *First-strand cDNA synthesis*

Recommended suppliers: Expand reverse transcriptase (Roche) or
Superscript II reverse transcriptase (Invitrogen)

3. *dNTPs Mix*

Recommended suppliers: dNTPs Mix (New England Biolabs)
or
dNTPs Mix (Invitrogen)

4. *Taq Polymerase*

Recommended suppliers: Platinum *Taq Polymerase*
or
High Fidelity PCR system (Roche)

Protocol:

[0188] Samples from patients suspected of having SARS infections, such as blood and tissue need to be handled according to WHO (on the world wide web at who.int/en/ as of April 20, 2004) or CDC (on the world wide web at cdc.gov/ as of April 20, 2004) recommendations.

1. RNA is extracted by manufacturers' instructions (TRIZOL® or Qiagen kits).

2. First-stand cDNA synthesis is performed on extracted RNA using GISSARSOUT_R (2 µl) in a 20 µl reaction volume.

3. First round PCR: The cDNA product (2.5 µl) is used as template using GISSARSOUT_F (1.25 µl) and GISSARSOUT_R (1.25 µl) in a 25 µl reaction volume. A 440 bp fragment can be detected at this step if the viral titre of the sample is very high. Note: The control templates (1 µl) can be run along side with every PCR reaction.

PCR conditions:

94°C for 2 min

94°C for 10 sec
50°C for 30 sec — 35 x cycles
72°C for 1 min

72°C for 7 min

4. Second round PCR: Products from the first round PCR (2.5 µl) is used as template for the second PCR using GISSARSIN_F (1.25 µl) and GISSARSIN_R (1.25 µl) in a 25 µl reaction volume with the same PCR conditions as the first round PCR. **Note: The control templates (1 µl) can be run along side with every PCR reaction.**

5. Products are analyzed on a 1.2 – 1.5% agarose gel. A 120 bp fragment can be detected for positive SARS confirmation. See Figure 6.

EXAMPLE 2: DETECTION OF SARS CORONAVIRUS BY REAL TIME PCR

[0189] SARS-CoV can be detected in a clinical sample using real time PCR rather than the standard RT-PCR method described in Example 1.

The following are mixed in a 20 µL reaction:

10 µL 2X Universal PCR Mix (Applied Biosystems)

1.8 µL 10 µM Primer 1 (e.g. SEQ ID NO: 11)

1.8 µL 10 µM Primer 2 (e.g. SEQ ID NO: 20)

0.5 µL 10 µM 5'-nuclease probe (e.g. SEQ ID NO: 21)

4.9 μL Water

1.0 μL template

[0190] Final concentrations of PCR primers are 900 nM; final concentration of 5'-nuclease probe is 250 nM.

[0191] Reactions are preferably run in duplicate.

[0192] PCR cycling is 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C, 15 seconds and annealing and extension at 60°C, 1 minute.

[0193] Fluorescence is monitored as described by the system manufacturer during annealing and extension cycles.

[0194] While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques and apparatus described above can be used in various combinations. All publications, patents, patent applications, and/or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, and/or other document were individually indicated to be incorporated by reference for all purposes.

APPENDIX

INTERNATIONAL PATENT APPLICATION For

REAGENTS AND METHODS FOR DETECTING SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS

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NOTES

Detection of Severe Acute Respiratory Syndrome Coronavirus in Blood of Infected Patients

Lisa F. P. Ng,[1] Michelle Wong,[2] Susie Koh,[2] Eng-Eong Ooi,[3] King-Fai Tang,[3] Hoe-Nam Leong,[4] Ai-Ee Ling,[5] Lora V. Agathe,[1] Jenny Tan,[1] Edison T. Liu,[1] Ee-Chee Ren,[1,6] Lee-Ching Ng,[2] and Martin L. Hibberd*[1]

Genome Institute of Singapore,[1] DSO National Laboratories,[2] Environmental Health Institute, National Environment Agency,[3] Tan Tock Seng Hospital,[4] Department of Pathology, Singapore General Hospital,[5] and Department of Microbiology, National University of Singapore,[6] Singapore, Republic of Singapore

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Severe acute respiratory syndrome (SARS) has caused major outbreaks worldwide, resulting in an urgent need to obtain sensitive and accurate diagnosis of this disease. PCR-based detection methods were developed for use on a variety of samples, including blood. Eighteen subjects were investigated, and results indicated that blood samples contain sufficient virus for detection by using quantitative real-time PCR.

Severe acute respiratory syndrome (SARS) was first identified in late November 2002 in Guangdong Province, China. In the ensuing months, major outbreaks were reported from Vietnam, Hong Kong, Canada, Singapore, other parts of China, Taiwan, and elsewhere in the world. The disease is unusual in its high level of infectivity, as demonstrated among the health care workers and family members that were in close contact with infected individuals. In addition, it was reported that infected patients do not respond to empirical antimicrobial treatment for acute community-acquired typical or atypical pneumonia (4, 7). The cause of SARS was identified as a novel coronavirus (CoV) (3, 4) because clinical specimens from patients infected with SARS revealed the presence of crown-shaped CoV particles. This new CoV was thus referred to as SARS CoV. The full-length genome sequence of the SARS CoV was reported from different isolates (5, 10), and the genome organization of SARS CoV was found to be similar to that of other CoVs (5, 10).

CoVs are a family of positive-strand RNA-enveloped viruses called *Coronaviridae*, which are now categorized under the newly established order *Nidovirales*. This order comprises the families *Coronaviridae* and *Arteriviridae*. The name *Nidovirales* comes from the Latin word nidus, for nest, referring to the 3′-coterminal "nested" set of subgenomic mRNAs produced during viral infection (2). The SARS CoV genome is very large, 29.7 kb (5, 10), and encodes 23 putative proteins. Major structural proteins include nucleocapsid, spike, membrane, and small envelope. Nonstructural proteins include the papain-like proteinase, 3C-like proteinase, RNA-dependent RNA polymerase (RdRp), helicase, and many other proteins involved with viral replication and transcription (2, 6). In other CoVs, many of the nonstructural proteins are only slightly conserved in the viral sequence, the exception being RdRp, which is highly conserved in many CoVs. In previous studies, primer pairs have been designed against different regions along the SARS CoV (3, 8, 9) and have managed to detect SARS CoV in a variety of clinical samples (3, 8, 9). In one report, the earliest detection observed was in sputum at day 3 (3), while in a different study, detection was found only at day 5 in nasopharyngeal aspirate (NPA), peaking at day 10 (8). In the same study, no association was found with the NPA viral load and clinical progression (8). For this report, we have investigated the use of blood as a means of detecting viral load in SARS patients, a method which in the future may allow improved estimation of disease progression.

Specific primers were designed against the highly conserved polymerase gene of the SARS CoV genome, as sequence comparison among the 14 SARS isolates demonstrated no variations in the SARS CoV RdRp region (10), making the RdRp an ideal region for designing specific diagnostic PCR primers in order to ensure that they will not quickly become obsolete due to sequence mutation. The performance of these primers was assessed in two assays using in vitro-transcribed RNA and virus-spiked samples. In the first PCR assay, 2 μl of RNA was reverse transcribed with Expand reverse transcriptase (Roche, Mannheim, Germany) using primer 5′-GGCATCATCAGAA AGAATCATCAT-3′, thereby generating 20 μl of cDNA. This was amplified with primers 5′-GGTTGGGATTATCCAAAA TGTGA-3′ and 5′-GGCATCATCAGAAAGAATCATCA T-3′ using 2.5 μl in 25-μl reaction mixtures (94°C for 10 s; 35 cycles of 94°C for 10 s, 50°C for 30 s, and 72°C for 1 min; and finally, 72°C for 7 min) with an Expand High-Fidelity PCR system (Roche). Nested primers (5′-ACTATATGTT

* Corresponding author. Mailing address: Genome Institute of Singapore, 60 Biopolis St., Genome, #02-01, Singapore 138672, Republic of Singapore. Phone: 65-647-88087. Fax: 65-647-89004. E-mail: hibberdml@gis.a-star.edu.sg.

TABLE 1. Investigation of subjects for presence of SARS CoV[a]

Subject no.	Diagnosis	Days from onset	Days from illness to discharge or result	Result of quantitative real-time PCR (copies/ml of blood)	Result of gel-based nested PCR
1	Probable SARS	2	9	2,020	+
2	Probable SARS	4	Died	9,100	+
3	Probable SARS	4	>30	3,120	+
4	Probable SARS	5	13	9,600	+
5	Probable SARS	12	18	Negative	−
6	Probable SARS	13	18	Negative	−
7	Probable SARS	14	>30	Negative	−
8	Probable SARS	14	36	Negative	−
9	Non-SARS	0	0	Negative	−
10	Non-SARS	2	1	Negative	−
11	Non-SARS	3	12	Negative	−
12	Non-SARS	10	9	Negative	−
13	Control			Negative	−
14	Control			Negative	−
15	Control			Negative	−
16	Control			Negative	−
17	Control			Negative	−
18	Control			Negative	−

[a] Eighteen subjects were tested by both nested PCR and quantitative real-time PCR (LightCycler). Four out of eight subjects with probable SARS cases tested positive. Specimens from non-SARS cases and controls from normal healthy individuals consistently tested negative.

AAACCAGGTGG-3′ and 5′-ATTTACATTGGCTGTAAC AGC-3′) were used in a second round of PCR, which used 2.5 μl of first-round PCR product as a template in a 25-μl reaction mixture. The size of this nested PCR product was 110 bp and was resolved in 1.5 to 2% agarose gels. PCR products were sequenced directly to confirm the identity of the products. Results are shown in Table 1.

Next, we used the LightCycler SARS CoV quantification kit (Roche) in a one-step reverse transcriptase PCR for the real-time quantitative PCR that was also designed for the polymerase region and utilized sequence-specific hybridization probes as the detection format. Five microliters of RNA was reverse transcribed and amplified in a 20-μl reaction mixture according to the manufacturer's recommendations.

In order to test the sensitivities of this assay, virus grown in Vero E6 cells was harvested, titrated, and spiked into 200 μl of Tris-EDTA buffer, sputum, stool suspension, and blood. RNA was extracted from spiked samples by using the HighPure nucleic acid viral kit (Roche) and the QIAamp viral RNA Mini kit (Qiagen) according to the manufacturers' instructions. Comparable results were achieved. Quantitated single-stranded RNA standards (provided by Roche) showed the sensitivity of the assay to be less than 85 copies per reaction (Fig. 1A). The melting curves (Fig. 1A, inset) also confirmed the specificity of the PCR. The internal control showed that there was no inhibition in the samples that had negative signals (Fig. 1B). The detected viral load in the spike samples varied from 1×10^3 to 5×10^3 copies per reaction (Fig. 1A). This suggests that the extraction protocol used was approximately 10% efficient, with sputum having the greatest efficiency for the clinical samples.

We noted that during the outbreak, most clinical specimens collected for SARS detection (including sputum, NPA, and endotracheal tube samples) involved considerable risk to the health care worker; thus, in order to reduce this risk, we have evaluated the suitability of blood for SARS CoV detection. We investigated 18 subjects comprising eight probable SARS patients and four patients who initially had symptoms similar to SARS but were later diagnosed otherwise. Two of the four subjects who were initially "suspect" for SARS were later diagnosed as having pulmonary tuberculosis and *Escherichia coli* urinary tract infection. The diagnosis of the other two patients was not determined. Four out of eight probable SARS patients had detectable virus in their blood, while the four non-SARS patients and six control samples from healthy individuals showed no virus (Fig. 2 and Table 1). In addition, control samples spiked with other human CoVs showed no signals (data not shown). Once again, melting-curve analysis confirmed the specificity of the PCR products (Fig. 2, inset). Viral load in the four positive SARS patients varied from 2×10^3 copies to 1×10^4 copies per ml of blood (Fig. 2; Table 1). It is unclear from these data whether this variation predicts clinical outcome; however, work from other studies has shown that blood viral load is a good indicator of disease progression (1). The two PCR methods described herein showed equal sensitivity in terms of detection. However, the gel-based assay method is laborious and nonquantitative.

We detected virus in blood at 2 days after the onset of symptoms, which is earlier than previously reported (3, 8, 9). Although it is important to further define the window period for detection of SARS CoV in blood, it is interesting to speculate that the time course of SARS CoV viremia may be relatively short, with our data suggesting that, at days 12 to 14, viral load is too low to detect (Table 1). More patients and sequential sampling would be required to confirm this supposition.

In conclusion, we have shown that the SARS CoV viral load can be determined in patients' blood by using PCR methods, and our data suggest that SARS CoV can be detected early in blood, i.e., within the first week of symptom onset. This fact allows early diagnosis and determination of viral load, both of

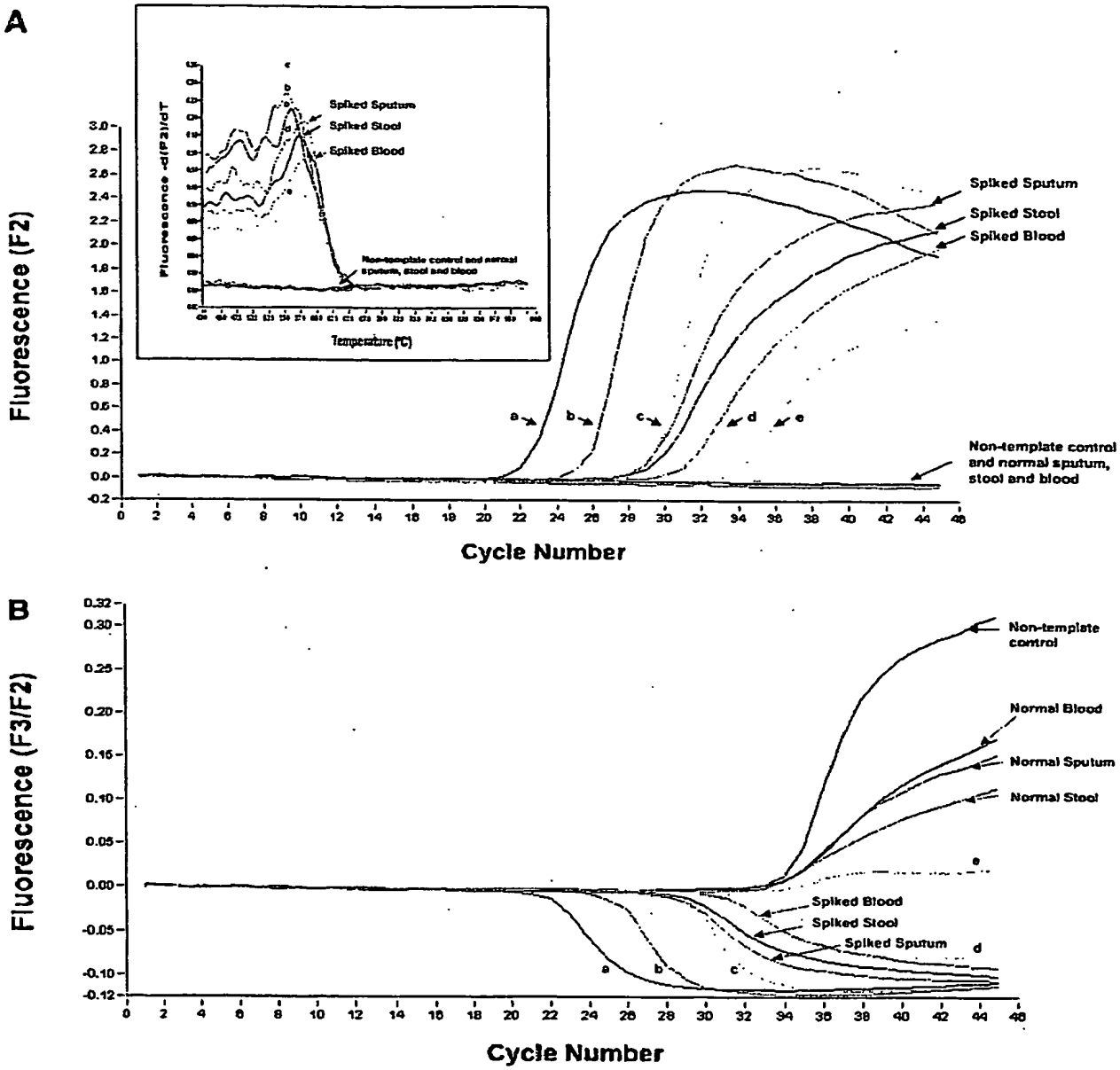

FIG. 1. Detection of SARS CoV by real-time quantitative PCR. (A) Amplification of single-stranded RNA standards (indicated as a to e) and RNA extracted from sputum, stool, and blood spiked with virus grown in Vero E6 cells. The *x* axis denotes the cycle number of the quantitative PCR assay, and the *y* axis denotes fluorescence intensity (F2) over the background level. The nontemplate control (water) is indicated. The viral load is indicated as the number of copies per reaction: spiked sputum, 5×10^5; spiked stool, 4×10^5; spiked blood, 1×10^5. RNA standards were as follows: (a) 1×10^6 copies per reaction, (b) 9.5×10^4 copies per reaction, (c) 8.7×10^3 copies per reaction, (d) 1.1×10^3 copies per reaction, and (e) 8.5×10 copies per reaction. The inset graph represents melting-curve analysis of the PCR products. Signals from RNA standards (a to e), spiked samples, normal samples, and nontemplate control (water) are shown. The *x* axis denotes the temperature (°C), and the *y* axis denotes the fluorescence intensity (F2) over the background level. (B) Detection of the internal control in fluorometer channel F3 in parallel with the simultaneous amplification of the RNA standards (a to e), spiked samples, and nontemplate control is shown.

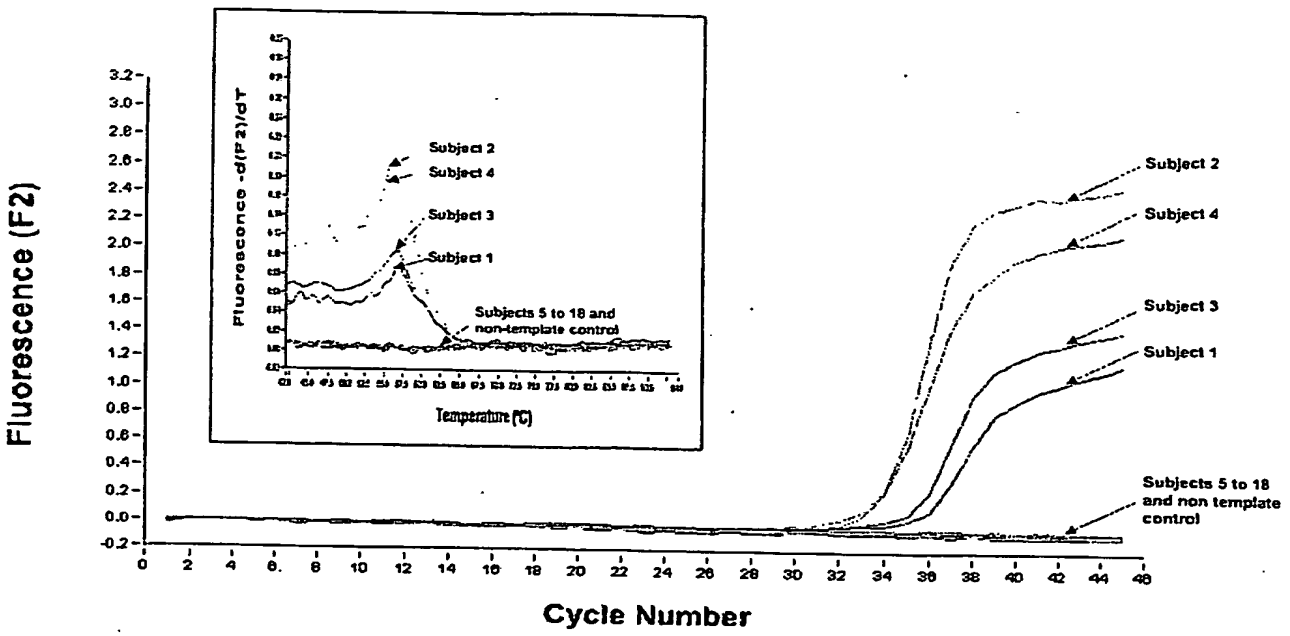

FIG. 2. Detection of SARS CoV from 18 subjects by real-time quantitative PCR. Data for RNA from 18 subjects are shown. The viral load is indicated as number of copies per ml of blood. Subject 1, 2,020 copies; subject 2, 9,100 copies; subject 3, 3,120 copies; subject 4, 9,600 copies. A sample would be considered positive if it generated a typical amplification curve within the 45 cycles. Negative signals from subjects 5 to 18 and the nontemplate control (water) are shown. The inset graph shows melting-curve analysis of the PCR products from the 18 subjects. The *x* axis denotes the temperature (°C), and the *y* axis denotes the fluorescence intensity (F2) over the background level.

which are useful in clinical and public health management settings. Further work is under way to further define the role of blood in diagnosis.

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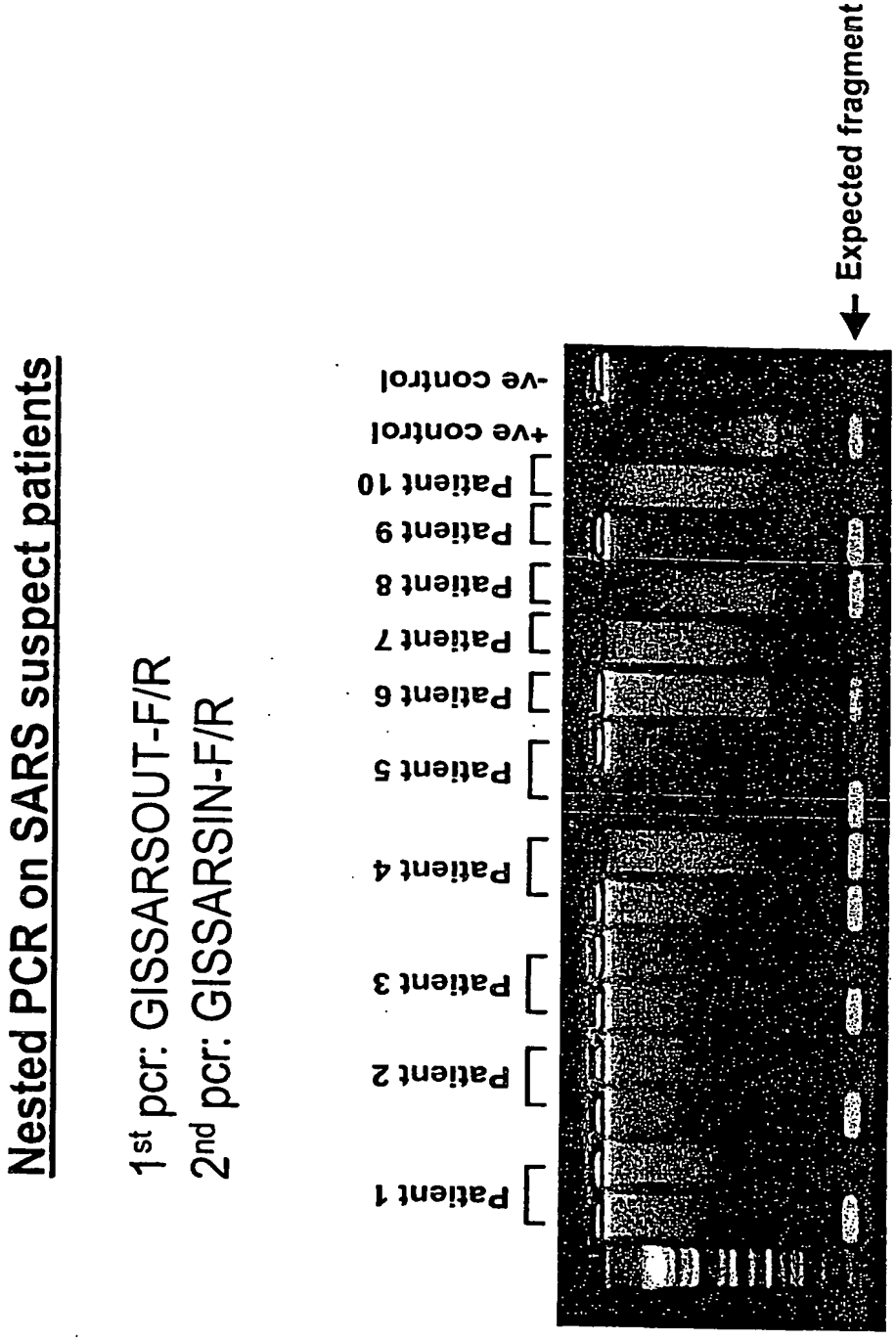
REFERENCES

1. Candotti, D., J. Temple, F. Sarkodie, and J. P. Allain. 2003. Frequent recovery and broad genotype 2 diversity characterize hepatitis C virus infection in Ghana, West Africa. J. Virol. 77:7914–7923.
2. Cavanagh, D. 2003. Nidovirales: a new order comprising *Coronaviridae* and *Arteriviridae*. Arch. Virol. 14:629–633.
3. Drosten, C., S. Gunther, W. Preiser, S. van der Werf, H. R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R. A. Fouchier, A. Berger, A. M. Burguiere, J. Cinatl, M. Eickmann, N. Escriou, K. Grywna, S. Kramme, J. C. Manuguerra, S. Muller, V. Rickerts, M. Sturmer, S. Vieth, H. D. Klenk, A. D. Osterhaus, H. Schmitz, and H. W. Doerr. 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N. Engl. J. Med. 348:1967–1976.
4. Kinzek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J. A. Comer, W. Lim, P. E. Rollin, S. F. Dowell, A. E. Ling, C. D. Humphrey, W. J. Shieh, J. Guarner, C. D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Y. Yang, N. Cox, J. M. Hughes, J. W. LeDuc, W. J. Bellini, L. J. Anderson, and the SARS Working Group. 2003. A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. 348:1953–1966.
5. Marra, M. A., S. J. M. Jones, C. R. Astell, R. A. Holt, A. Brooks-Wilson, Y. S. Butterfield, J. Khattra, J. K. Asano, S. A. Barber, S. Y. Chan, A. Cloutier, S. M. Coughlin, D. Freeman, N. Girn, O. L. Griffith, S. R. Leach, M. Mayo, H. McDonald, S. B. Montgomery, P. K. Pandoh, A. S. Petrescu, A. G. Robertson, J. E. Schein, A. Siddiqui, D. E. Smailus, J. M. Stott, G. S. Yang, F. Plummer, A. Andonov, H. Artsob, N. Bastien, K. Bernard, T. F. Booth, D. Bowness, M. Czub, M. Drebot, L. Fernando, R. Flick, M. Garbutt, M. Gray, A. Grolla, S. Jones, H. Feldmann, A. Meyers, A. Kabani, Y. Li, S. Normand, U. Stroher, G. A. Tipples, S. Tyler, R. Vogrig, D. Ward, B. Watson, R. C. Brunham, M. Krajden, M. Petric, D. M. Skowronski, C. Upton, and R. L. Roper. 2003. The genome sequence of the SARS-associated coronavirus. Science 300:1399–1404. (First published online 1 May 2003; 10.1126/science.1085953.)
6. Ng, L. F. P., and D. X. Liu. 2002. Membrane association and dimerization of a cysteine-rich, 16-kilodalton polypeptide released from the C-terminal region of the coronavirus infectious bronchitis virus 1a polyprotein. J. Virol. 76:6257–6267.
7. Peiris, J. S. M., S. T. Lai, L. L. Poon, Y. Guan, L. Y. Yam, W. Lim, J. Nicholls, W. K. Yee, W. W. Yan, M. T. Cheung, V. C. Cheng, K. H. Chan, D. N. Tsang, R. W. Yung, T. K. Ng, K. Y. Yuen, and the SARS Study Group. 2003. Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet 361:1319–1325.
8. Peiris, J. S. M., C. M. Chu, V. C. C. Cheng, K. S. Chan, I. F. N. Hung, L. L. Poon, K. I. Law, B. S. F. Tang, T. Y. W. Hon, C. S. Chan, K. H. Chan, J. S. C. Ng, B. J. Zheng, W. L. Ng, R. W. M. Lai, Y. Guan, K. Y. Yuen, and the HKU/UCH SARS Study Group. 2003. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. Lancet 361:628–633.
9. Poon, L. L. M., O. K. Wong, W. Luk, Y. Y. Kwok, J. S. M. Peiris, and Y. Guan. 2003. Rapid diagnosis of a coronavirus associated with severe acute respiratory syndrome (SARS). Clin. Chem. 49:953–955.
10. Ruan, Y. J., C. L. Wei, A. E. Ling, V. B. Vega, H. Thoreau, S. Y. Se Thoe, J. M. Chia, P. Ng, K. P. Chiu, L. Lim, T. Zhan, K. P. Chan, L. L. E. Ooi, M. L. Ng, Y. S. Leo, L. F. P. Ng, E. C. Ren, L. W. Stanton, P. M. Long, and E. T. Liu. 2003. Comparative full-length genome sequence analysis of 14 SARS coronavirus isolates and common mutations associated with putative origins of infection. Lancet 361:1779–1785.


Nested PCR on SARS suspect patients
1st pcr: GISSARSOUT-F/R
2nd pcr: GISSARSIN-F/R
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
Patient 6
Patient 7
Patient 8
Patient 9
Patient 10
+Ve control
-Ve control
Expected fragment

Annex: Protocol for LightCycler RNA Master SYBR Green

Protocol:

1. 2 µl of sample RNA is used for each reaction. **Note: The positive control template (1 µl) should be run along. A negative control should also be run with the samples.**
2. Reagent preparation should be done in either 0.2 ml or 1.5 ml reaction tubes on ice added in the following order:

Component	Volume
Sterile water	X µl
$Mn(OAc)_2$ stock solution, 50 mM	1.3 µl
LC F / R Mix	2 µl
Template	X µl
LightCycler RNA Master SYBR Green I	7.5 µl

Mix gently and transfer reaction mix to glass capillaries.

Program 1: Reverse Transcription

The recommended T°C for the RT step is 61°C.

Cycle Prog Data	Value
Cycles	1
Analysis Mode	None
Temperature Targets	**Segment 1**
Target T°C	61
Incubation time	20 min
T°C transition rate	20
Secondary target T°C	0
Step size (°C)	0.0
Step Delay (cycles)	0
Acquisition mode	None

Program 2: Denaturation

Cycle Prog Data	Value
Cycles	1
Analysis Mode	None
Temperature Targets	**Segment 1**
Target T°C	95
Incubation time	10 sec
T°C transition rate	20
Secondary target T°C	0
Step size (°C)	0.0
Step Delay (cycles)	0

	Acquisition mode	None

Program 3: Amplification

Cycle Prog Data		Value	
Cycles		40	
Analysis Mode		Quantification	
Temperature Targets	**Segment 1**	**Segment 2**	**Segment 3**
Target T°C	95	50	72
Incubation time	16 sec	16sec	16 sec
T°C transition rate	20	20	2
Secondary target T°C	0	0	0
Step size (°C)	0.0	0.0	0.0
Step Delay (cycles)	0	0	0
Acquisition mode	None	None	Single

Program 4: Melting Curve Analysis

Cycle Prog Data		Value	
Cycles		1	
Analysis Mode		Melting Curve	
Temperature Targets	**Segment 1**	**Segment 2**	**Segment 3**
Target T°C	95	60	95
Incubation time	5 sec	15 sec	0 sec
T°C transition rate	20	20	0.1
Secondary target T°C	0	0	0
Step size (°C)	0.0	0.0	0.0
Step Delay (cycles)	0	0	0
Acquisition mode	None	None	Cont.

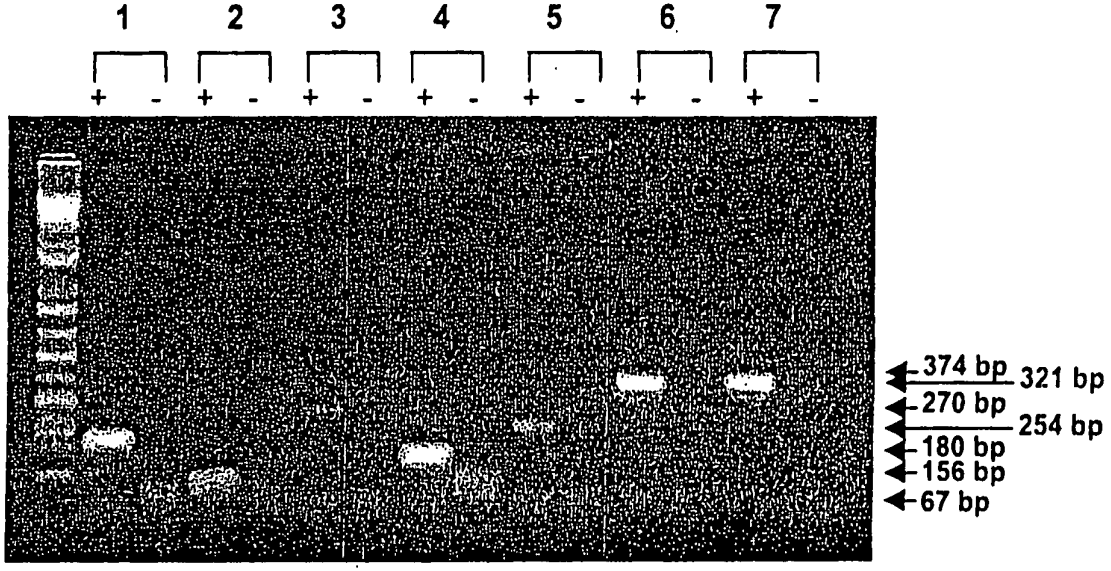
Program 5: Cooling

Cycle Prog Data	Value
Cycles	1
Analysis Mode	None
Temperature Target	**Segment 1**
Target T°C	40
Incubation time	30 sec
T°C transition rate	20
Secondary target T°C	0
Step size (°C)	0.0
Step Delay (cycles)	0
Acquisition mode	None

Annex: LightCycler RNA Master SYBR Green Detection on different primer combinations

- Set 1: 1 and 4 – 180 bp *
- Set 2: 1 and 2 – 67 bp *
- Set 3: 1 and 6 – 270 bp
- Set 4: 3 and 4 – 156 bp *
- Set 5: 3 and 6 – 254 bp
- Set 6: 3 and 7 – 374 bp *
- Set 7: 5 and 7 – 321 bp *

Primer 1 (SEQ ID NO: 23)
Primer 2 (SEQ ID NO: 19)
Primer 3 (SEQ ID NO: 18)
Primer 4 (SEQ ID NO: 20)
Primer 5 (SEQ ID NO: 15)
Primer 6 (SEQ ID NO: 16)
Primer 7 (SEQ ID NO: 12)



The LightCycler RNA Master SYBR Green 1 (3064760) was used to test the sensitivity of the primers described herein. The template used was RNA isolated from serum "spike" with virus from Vero isolates. For primer set 1, 4, 6 and 7 signals showed up between cycles 17 to 20. The same RNA template was also done on the Artus kit and signals showed up only after cycle 23.

Program 1: Reverse Transcription
Program 2: Denaturation – Incubation time changed to 10 sec
Program 3: Amplification – Incubation time changed to 16 sec

Annex: Results of LightCycler RNA Master SYBR Green with GIS SARS Primers

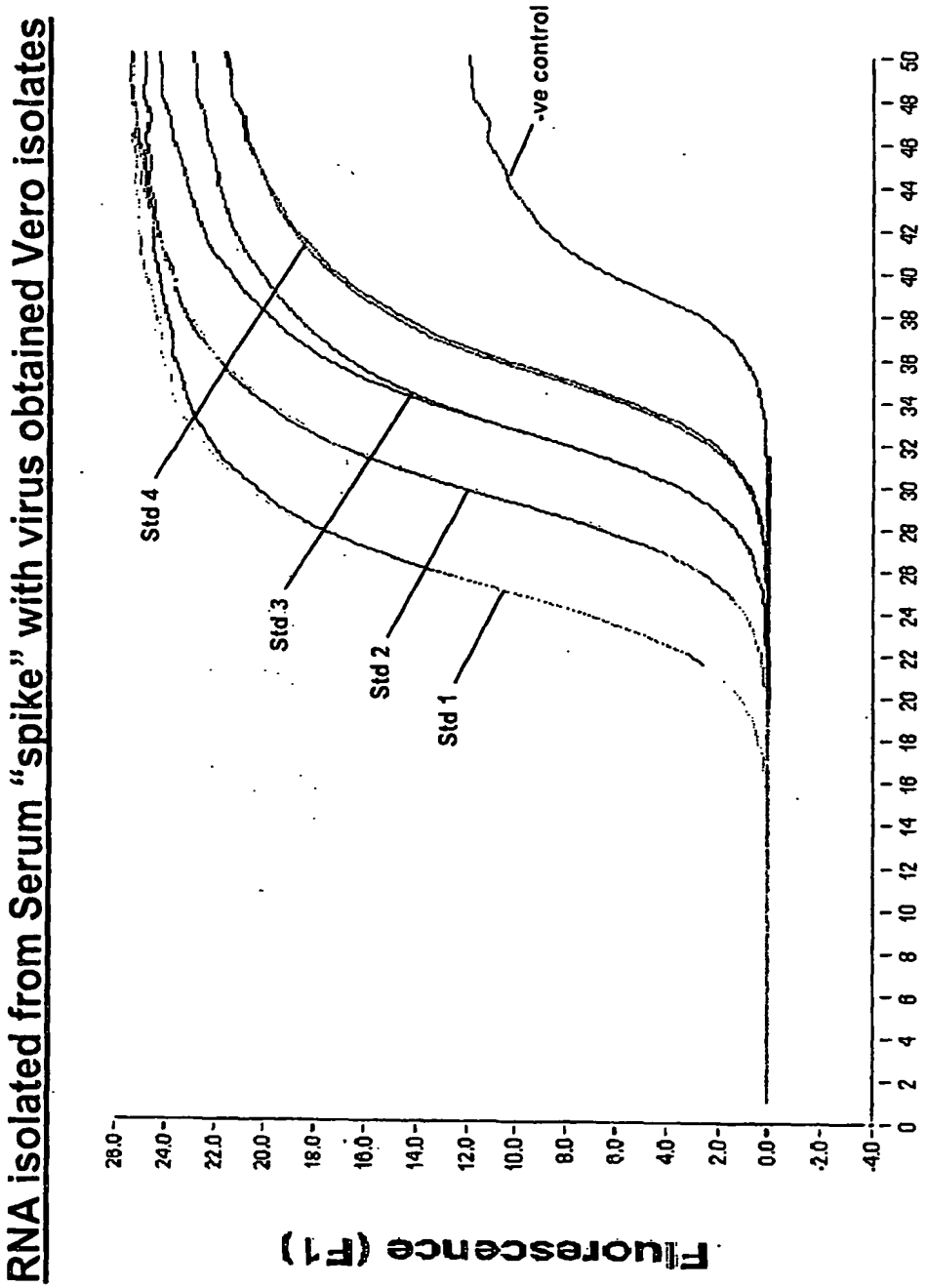


Fig. 1

Melting curve for RNA isolated from "spike" serum

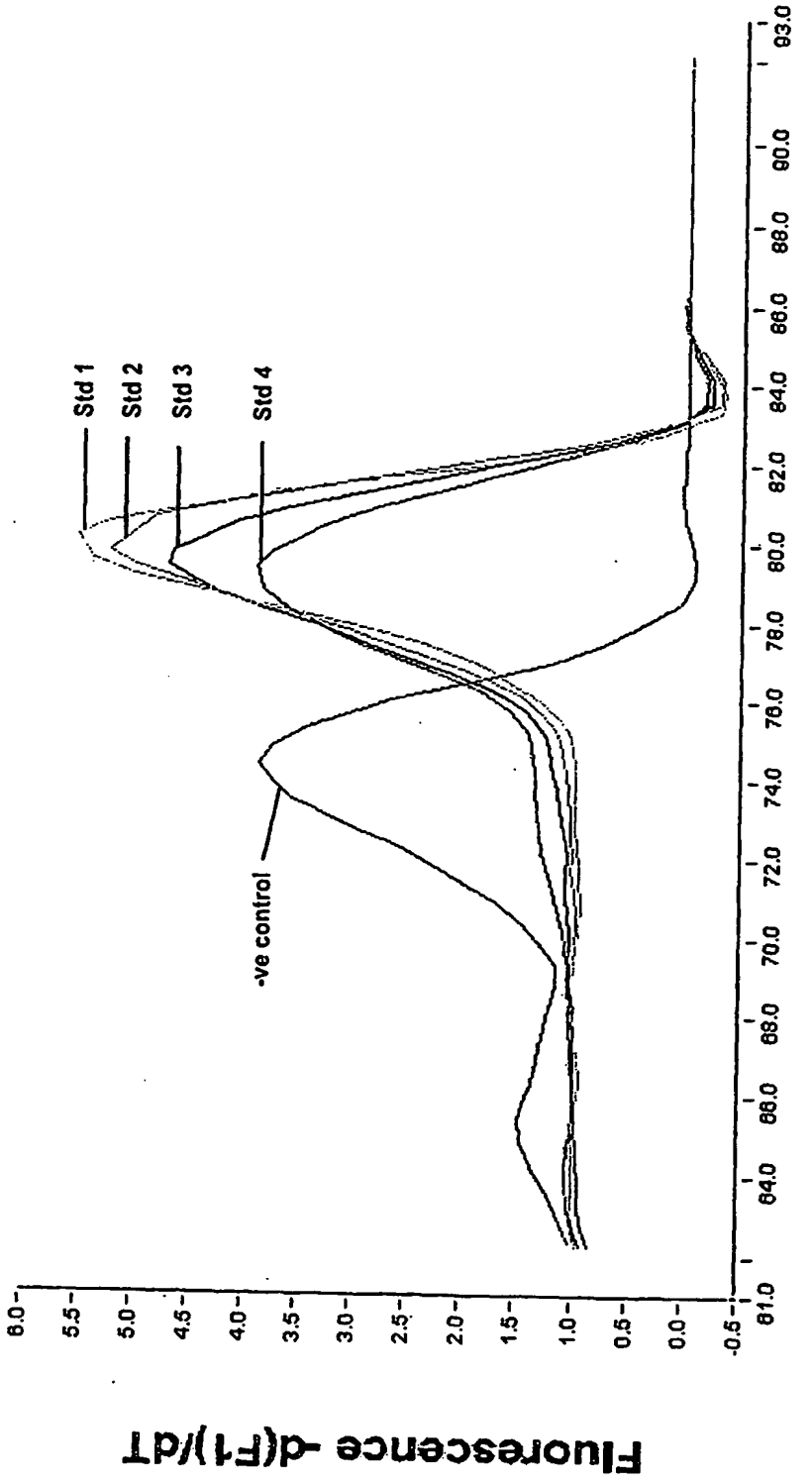


Fig. 1a

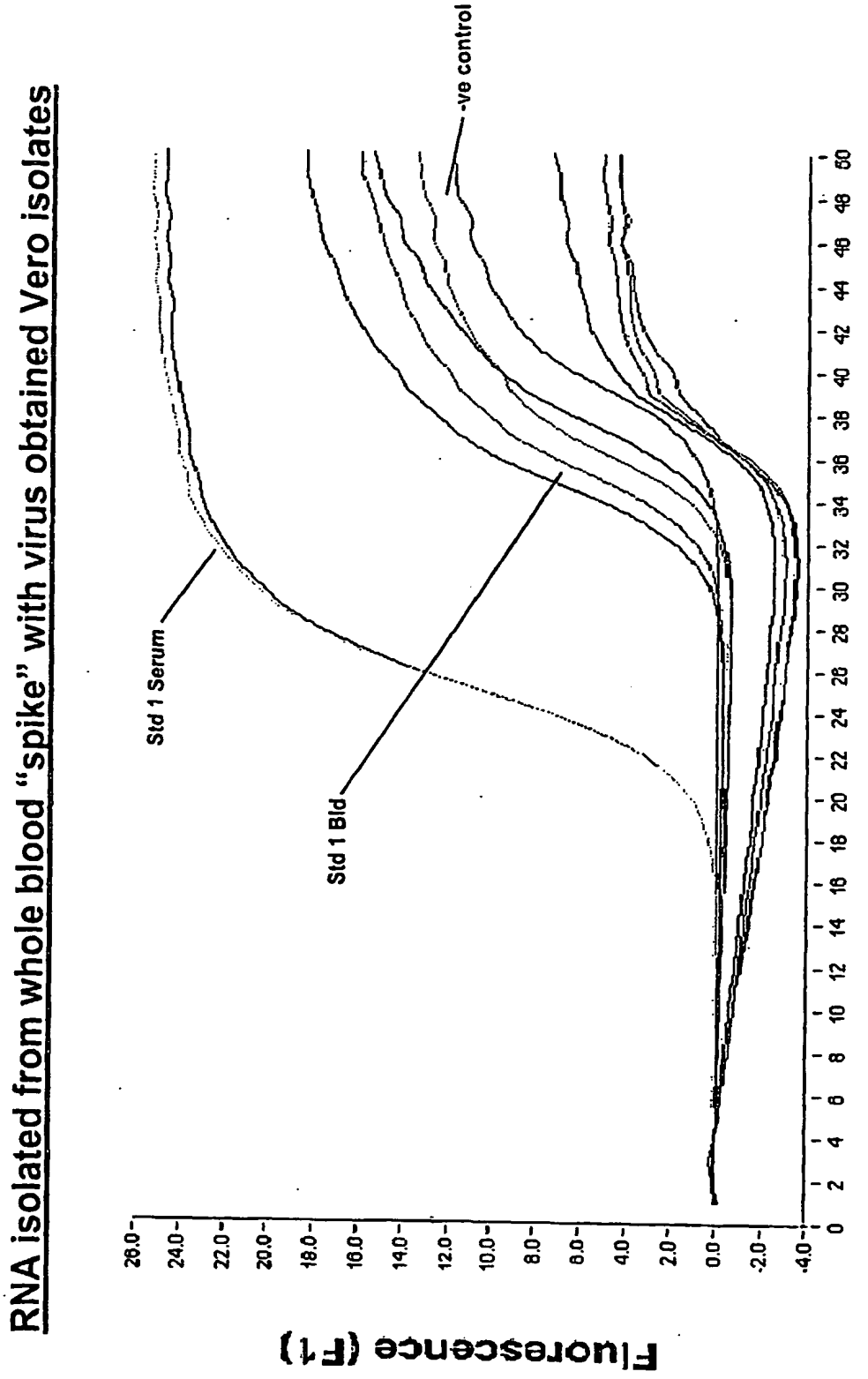

RNA isolated from whole blood "spike" with virus obtained Vero isolates
-ve control
Std 1 Serum
Std 1 Bld
Fluorescence (F1)
Cycle Number
Legend:
Std 1 Bld: RNA Standard diluted 1 x 10¹
Std 2 Bld: RNA Standard diluted 1 x 10²
Std 1 Serum: RNA standard diluted 1 x 10¹

Fig. 2

SARS-CoV Detection RT-PCR Diagnostic Using 5'-Nuclease Probe on the LightCycler

Specifications:

The SARS-CoV Detection RT-PCR is specifically designed for detecting patients suspected of having SARS using the LightCycler RNA Master Hybridization Probes (*"Hot Start"*) using a specially designed 5'-Nuclease Probe. This system has been adapted to be used in the LightCycler. Our results to date have been performed on RNA extracted from both whole blood (Trizol) and serum (Qiagen viral kit). Data showed that both extraction methods are fairly comparable, indicating that Trizol do not inhibit the PCR reactions. For sensitivity, this system is able to detect as low as 10 copies at cycle 35.

Protocol:

1. Reagent preparation should be done in 1.5 ml reaction tubes on ice added in the following order:

Component	Volume
Sterile water	X µl
$Mn(OAc)_2$ stock solution, 50 mM	1.3 µl
LC-ProbeNPrimer Mix	2.6 µl
LightCycler RNA Master Hybridization probes	7.5 µl

Mix gently and transfer reaction mix to glass capillaries.

2. 5 µl of sample RNA is added to each glass capillary.
3. The cycle program is as shown.

Program 1-Reverse Transcription

Cycle Program Data	Value
Cycles	1
Analysis Mode	None
Temperature Targets	**Segment 1**
Target T°C	61
Incubation time	20 min
T°C transition rate (°C/s)	20.0
Secondary Target T°C	0
Step Size (°C)	0.0
Step Delay (cycles)	0
Acquisition Mode	None

Program 2-Denaturation

Cycle Program Data	Value
Cycles	1
Analysis Mode	None

Temperature Targets	Segment 1
Target T°C	95
Incubation time	2 min
T°C transition rate (°C/s)	20.0
Secondary Target T°C	0
Step Size (°C)	0.0
Step Delay (cycles)	0
Acquisition Mode	None

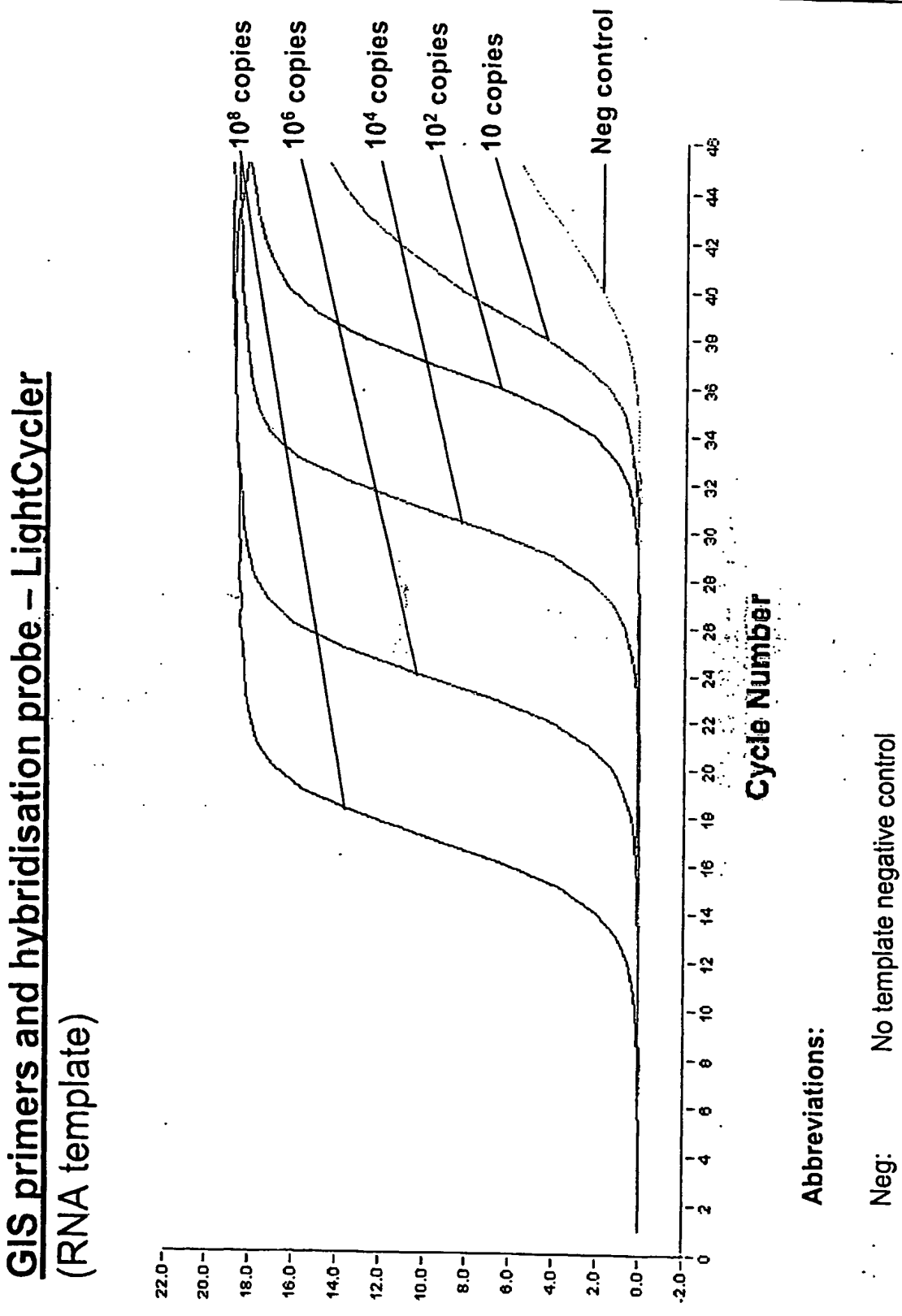
Program 3-Amplification

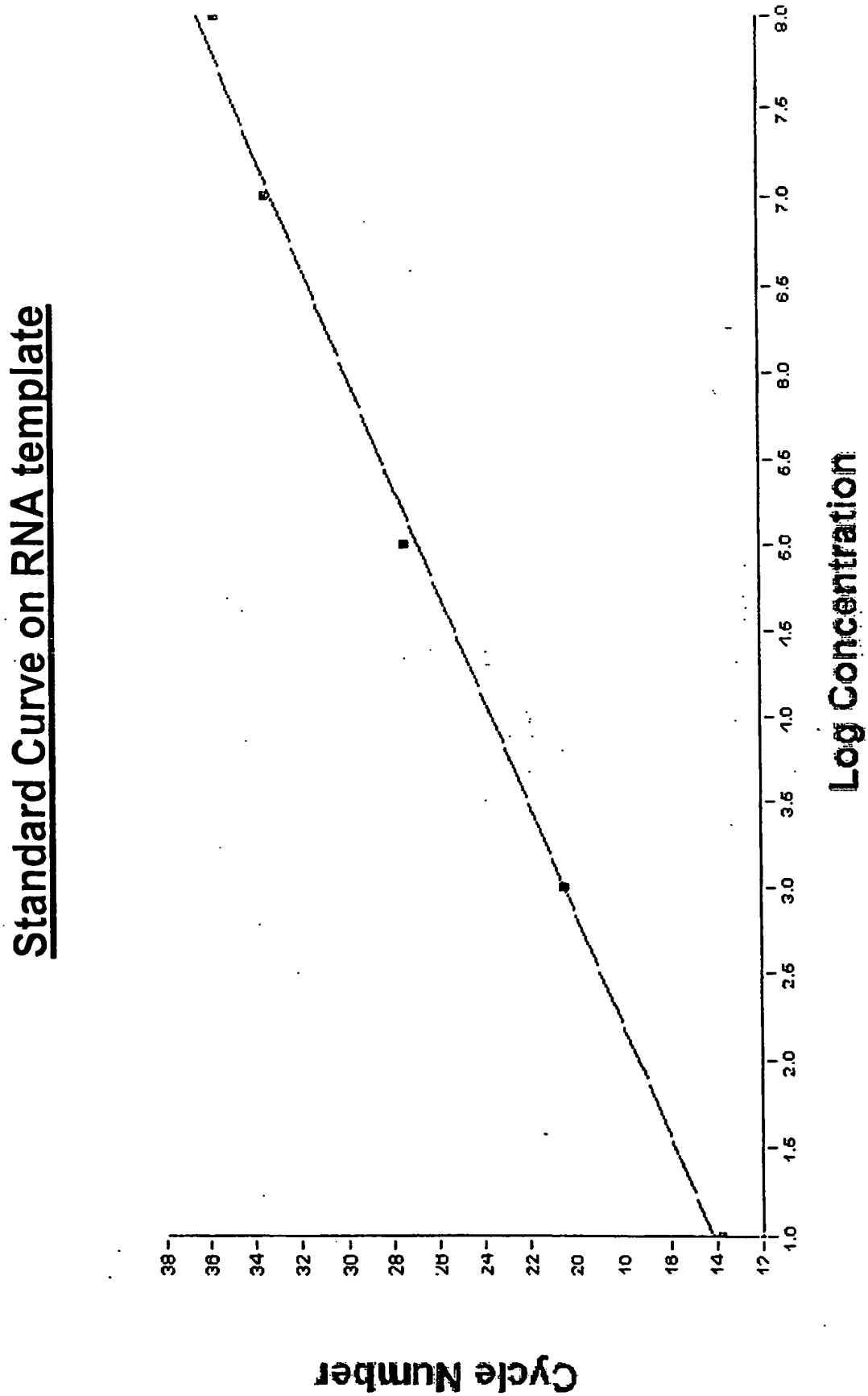
Cycle Program Data	Value	
Cycles	1	
Analysis Mode	Quantification	
Temperature Targets	**Segment 1**	**Segment 2**
Target T°C	95	60
Incubation time	5 sec	1 min
T°C transition rate (°C/s)	20.0	20.0
Secondary Target T°C	0	0
Step Size (°C)	0.0	0.0
Step Delay (cycles)	0	0
Acquisition Mode	None	Single

Program 4-Cooling

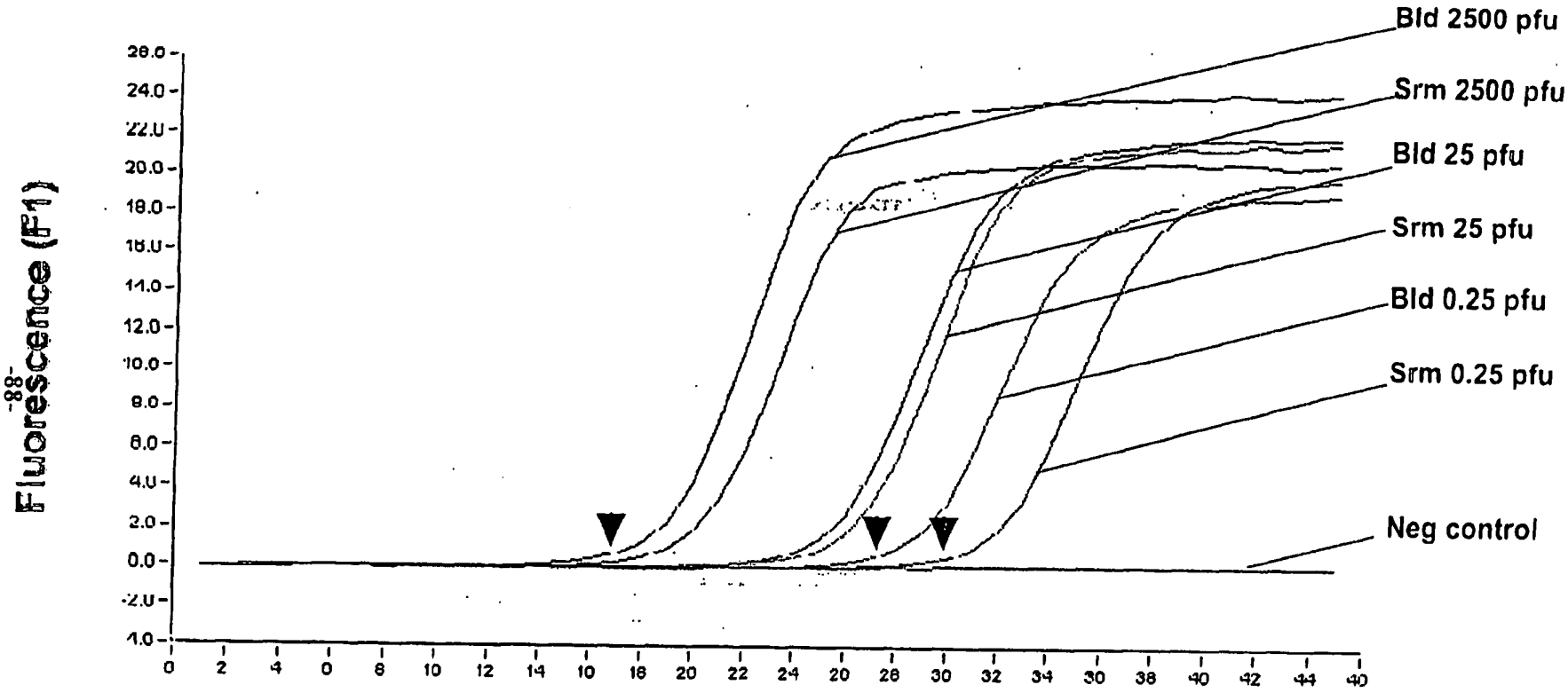
Cycle Program Data	Value
Cycles	1
Analysis Mode	None
Temperature Targets	**Segment 1**
Target T°C	40
Incubation time	30 sec
T°C transition rate (°C/s)	20.0
Secondary Target T°C	0
Step Size (°C)	0.0
Step Delay (cycles)	0
Acquisition Mode	None

Annex: Results of 5'-Nuclease Probe in LightCycler using GISSARS Primers




Standard Curve on RNA template
Cycle Number
Log Concentration
38
36
34
32
30
28
26
24
22
20
18
16
14
12
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0

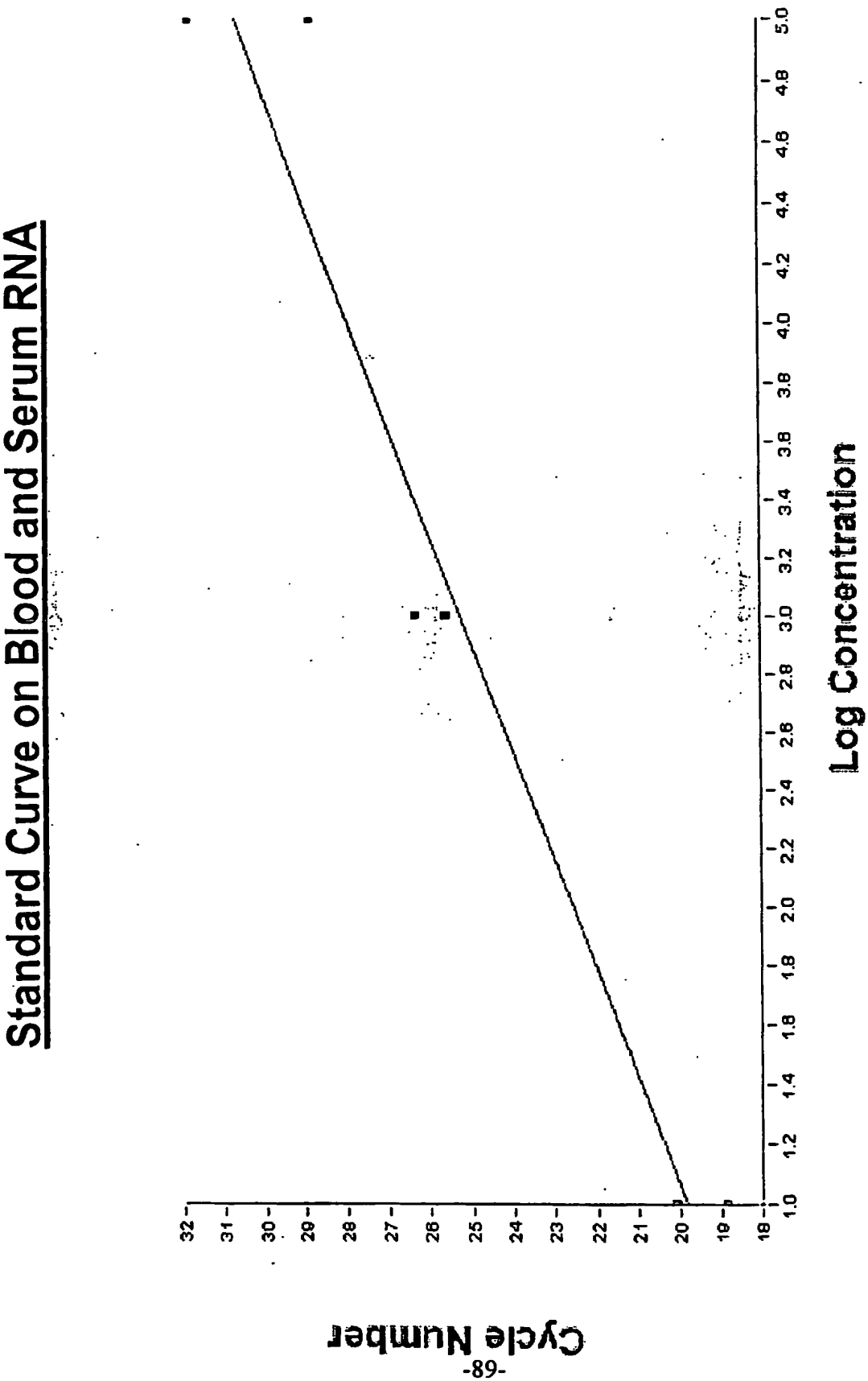
GIS primers and hybridisation probe – LightCycler



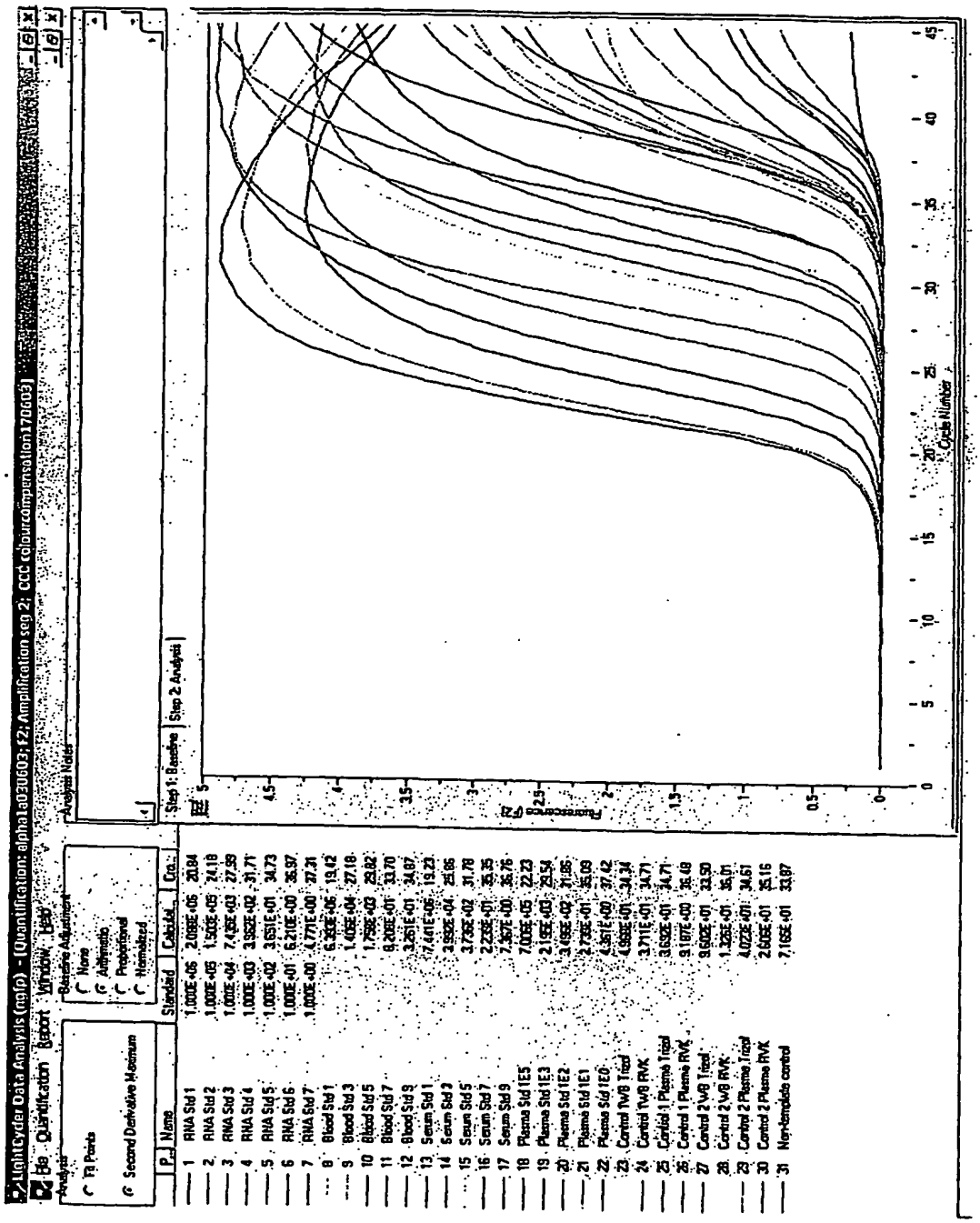
Abbreviations:

Bld:	RNA isolated from "spike" blood
Srm:	RNA isolated from "spike" serum
Neg:	No template negative control

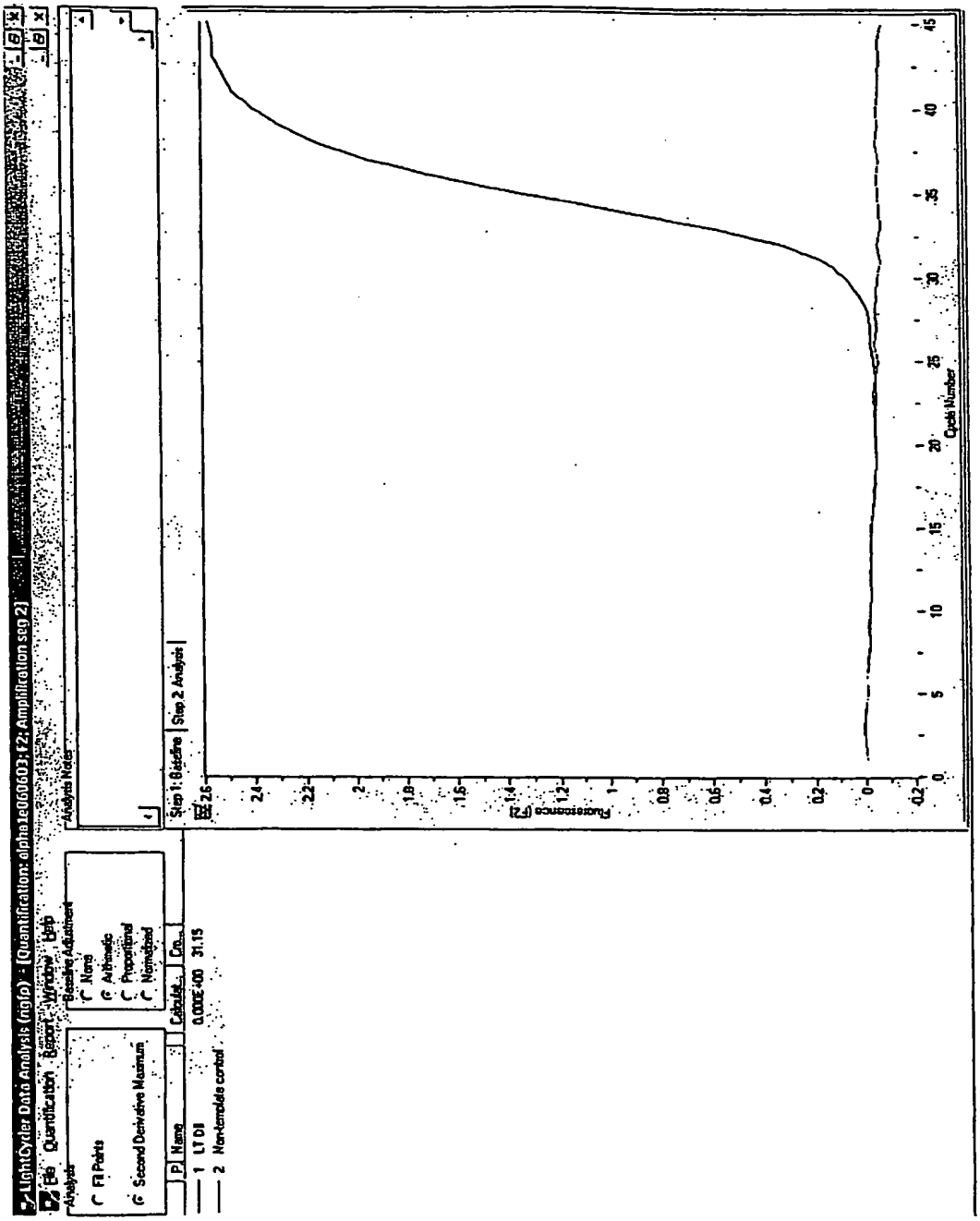
Signals started showing at cycle 17 and can detect 0.25 particles from blood at Cycle 27!


Standard Curve on Blood and Serum RNA
Cycle Number
32
31
30
29
28
27
26
25
24
23
22
21
20
19
18
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Log Concentration

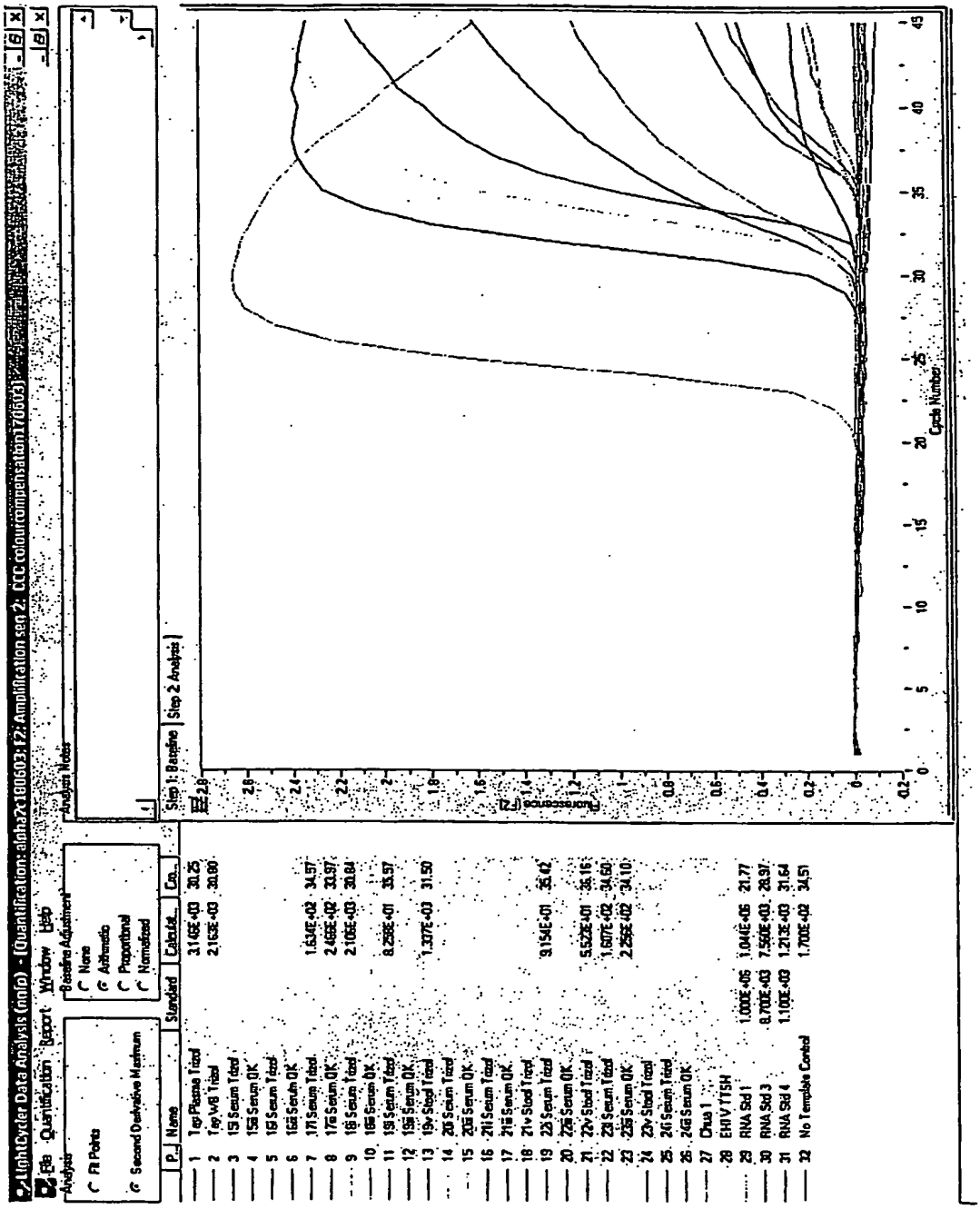
Test with RNA Standards and Test Samples



Test with Positive Control Sample



Test with RNA Standards and Test Samples:
(Quantitation curves indication viral load)



Test with RNA Standards and Test Samples:
(Melting curves indicating specificity of PCR)